The Genetic and Physical Analysis of Mutants of Chloroplast Number and Size in Arabidopsis thaliana

Thesis

The Genetic and Physical Analysis of Mutants of Chloroplast Number and Size in *Arabidopsis thaliana*

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by

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ABSTRACT

The genetic control of chloroplast division in *Arabidopsis thaliana* was investigated by the study of ***ARC*** genes. Seven ***arc*** mutants which displayed altered mesophyll cell chloroplast number and size compared to wild type were isolated from populations of *Arabidopsis* mutagenised by T-DNA or transposon insertion. The *arc* mutants represent alleles of six independent nuclear loci and are stably inherited in a normal Mendelian manner. The mean chloroplast number per mesophyll cell in *arc* mutants and wild type varies between 2 and 120 chloroplasts per cell. The size of *arc* mutant chloroplasts varies between half the size to 50 fold larger than wild type. Chloroplast number and chloroplast size varies inversely in the *Arabidopsis* mesophyll cell so that total chloroplast cover per cell is similar between wild type and *arc* mutants. The *arc* mutants are fully fertile and only one mutant, *arc*6, displays any significant whole plant mutant phenotype.

The chloroplasts of ***arc*5** do not normally divide but expand to a plan area six fold larger than wild type. Almost all *arc*5 chloroplasts are constricted around the centre of the plastid, indicating that chloroplast division has been attempted but not completed. The comparison of the chloroplast phenotype of *arc*5 to wild type has suggested several physical constraints to chloroplast division in *Arabidopsis* mesophyll cells.

***arc*11**, which has highly variable chloroplast number and size per mesophyll cell, is tagged by the insertion of a transposon, indicated by reversion of the *arc*11 mutant phenotype to wild type. Fragments of the putative *ARC*11 gene have been amplified by IPCR, cloned and sequenced.

The ***arc*6** mutant which affects both proplastid and chloroplast division may have as few as a single chloroplast per cell. *arc*6 is not tagged, requiring a map-based strategy to isolate *ARC*6. RFLP analysis using the **ARMS** and **CAPS** mapping sets indicated that *ARC*6 is located on chromosome 5, between markers m247 and DFR.

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ABBREVIATIONS

ΔNae*I *Ac	*Nae*I deleted mobile *Ac*
***ΔNae*I sAc**	*Nae*I deleted stable *Ac*
0383	*Ac* recombinant plasmid No. 0383
02213	*Ac* recombinant plasmid No. 02213
ABRC	Arabidopsis Biological Resource Centre, Ohio.
Ac	*Activator*
arc	Accumulation and replication of chloroplasts
***arc*X**	Mutant of gene "*ARCX*"
***arc*X-2**	Second mutant allele of gene "*ARCX*"
ARCX	Gene *No.*X of Accumulation and Replication of Chloroplasts
ARCX	Protein product of gene "*ARCX*"
ARMS	*Arabidopsis* RFLP Mapping Set
bp.	nucleotide base pairs
CAPS	Co-Amplified Polymorphic Sequences
cDNA	Complimentary DNA
Col	Columbia ecotype of *Arabidopsis*
CTAB	Mixed alkyl tri-methylammonium bromide
CtDNA	Chloroplast DNA
cv.	Cultivar
DAPI	4,6-diamidino-2-phenylindole
DIG	Dioxygenin
DNA	Deoxyribonucleic acid
Ds	*Dissociation*
E.coli	*Escherichia-coli*
EMS	Ethyl-methane sulphonate
En/Spm	*Enhancer-Inhibitor/Suppressor-mutator*
FG	Full Green
IPCR	Inverse Polymerase Chain Reaction
kanR	Kanamycin Resistant
kanS	Kanamycin Sensitive
kb.	Kilobase pairs
LB	Left Border
Ler	Landsberg *erecta* ecotype of *Arabidopsis*
LiAc	Lithium Acetate
Mb.	Megabase pairs
NaAc	Sodium Acetate
NASC	Nottingham *Arabidopsis* Stock Centre

NcDNA	Nuclear DNA
NOS	Nopaline Synthetase
NPTII	Neomycin phosphotransferase(kanR in bacteria)
1' NPTII	Neomycin phosphotransferase (kanR in plants)
OD	Optical density
PCR	Polymerase Chain Reaction
PEG	Poly ethalene glycol
PtDNA	Plastid DNA
RB	Right border
RAPD	Random Amplified Polymorphic Sequences
rbcL	Rubisco larger subunit gene
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
SPT	Streptomycin phosphotransferase
SSC	Sodium sodium citrate
TBE	Tris borate EDTA
T-DNA	Transfer DNA of *Agrobacterium tumefaciens*
TE	Tris EDTA
TEM	Transmission Electron Microscope.
Ti	Tumour-inducing gene of T-DNA
tRNA	Transfer RNA
VG	Variegated Green
WS	Ecotype Wassilewskija of *Arabidopsis*
WT	Wild type
YAC	Yeast Artificial Chromosome

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CHAPTER 1

Introduction

1.1 The study of chloroplast division

The attainment of photosynthetic competence by the leaf is essential to its development and efficient function in higher plants. The accumulation of the photosynthetic cell's complement of chloroplasts is essential to this process, providing the optimum photosynthetic efficiency for the cell. The mesophyll cell develops its chloroplast complement during cell development in the expanding leaf by the replication and expansion of chloroplasts derived from a small number of undifferentiated proplastid initials. The concept that chloroplasts arise by the replication of existing plastids rather than by *de novo* synthesis was first proposed by Schimper in 1885. This observation was later supported by Granick (1955) who observed that chloroplast number increased in the cells of expanding tomato leaves; also Fazze-franzeisket (1955) observed a six fold increase in plastid number per cell during differentiation of the leaves of *Agapanthus umbellatus*. The mechanisms of chloroplast division and the factors controlling the process were first investigated in higher plants by Possingham and Saurer (1969); Boasson and Laetsch (1969) and Ridley and Leech (1970). Further study of constricted chloroplasts by light and electron microscopy strengthened the suggestion that the majority of higher plant chloroplasts arise by the division of young green chloroplasts rather than *de novo* synthesis or the replication of proplastids.

The precise nature of the chloroplast division process is still unknown, although the sequence of events observed by light and electron microscopy have been well documented (Leech, Thomson and Platt-Aloia, 1981; Possingham and Lawrence, 1983; Whatley, 1988). The analysis of the mechanisms and control of chloroplast division and accumulation is central to the aims of this thesis.

The replication and accumulation of the chloroplast complement is of significant importance to the development of the mesophyll cell. In most species of higher plant the chloroplast population represents the major cytoplasmic compartment of the photosynthetic cell. An understanding of the development of the chloroplast complement of the mesophyll cell is therefore anticipated to aid the understanding of mesophyll cell development as a whole. Furthermore, the development of the chloroplast complement is

suggested to be significantly associated with the differentiation of the mesophyll cell from the undifferentiated post-meristematic cells. A better understanding of the process of chloroplast division and development is therefore likely to aid the study of the differentiation of the mesophyll cells.

The chloroplasts of higher plants contain their own DNA which is functionally integrated with the DNA of the nuclear genome so that genes which affect chloroplast division and growth may be contained in the nuclear genome as well as in the CtDNA (Douce and Joyard, 1984).

The study of chloroplast biology is therefore integral to the study of mesophyll cell development. The further understanding of the process of chloroplast division and the accumulation of the chloroplast complement is likely to aid the analysis of the development of the cell as a whole.

1.2 Plastid development in higher plants

The higher plant chloroplast develops in young mesophyll cells from the small plastid initials known as *proplastids* (Kirk and Tilney-Bassett, 1978; Leech 1984, 1986). The proplastids are typically present in meristematic cells and the proplastid population divides in association with cell division (Possingham, 1980). The replication of the proplastids in the dividing cells of the meristem maintains plastid continuity during cell division so that all cells will eventually contain plastids. The proplastids are undifferentiated and contain only very few rudimentary thylakoid membranes which contain only a little chlorophyll and are present as perforated plates, occasionally fused together (Leech, 1986). Proplastids may differentiate during the determination of cell types into the photosynthetic chloroplasts; amyloplasts of the root; or other plastids such as chromoplasts or leucoplasts (Kirk and Tilney-Bassett, 1978). Differentiating chloroplasts are often observed to accumulate starch before becoming amoeboid in their morphology (Whatley, 1979; Leech, 1983, 1986). The thylakoid membranes then proliferate before the plastid develops into a chloroplast (Leech, 1986). The young chloroplast which has differentiated from a proplastid in the cells of the leaf rapidly develops during the formation of the leaf primordia and the subsequent expansion and

growth of the leaf itself. The internal thylakoid membrane matrix develops and the photosynthetic complexes are integrated into the thylakoids (Whatley, Hawes, Horne and Kerr, 1982). As development proceeds, stacking of the thylakoids into grana is observed (Leech, 1986). It is during the development of the young chloroplasts that chloroplast division is observed to occur (Leech, 1984). Division of the chloroplast proceeds by the constriction of the chloroplast into two smaller daughter plastids; the process of division in higher plants is discussed in detail below. The division of the chloroplast in wheat (*Triticum aestivum* cv. Maris dove) (Boffey, Ellis, Sellden and Leech, 1979) is preceded by the replication of the CtDNA within the chloroplast (Boffey and Leech, 1982; Marrison and Leech, 1992). The wheat chloroplasts are observed to maintain a constant ratio of CtDNA to NcDNA amount in subsequent chloroplast development after CtDNA replication has occurred (Bowman, 1986). The chloroplast division cycle proceeds during mesophyll cell development until the final complement of chloroplast number per cell is achieved. The number of chloroplasts per mesophyll cell is very conserved within ecotypes of species (Pyke and Leech, 1987). The fully developed mesophyll cell chloroplast therefore has undergone two periods in its history where plastid division affects its development - the replication of proplastids and the division of maturing, green chloroplasts. The control of the process of chloroplast division therefore has a significant effect on the development of the chloroplast complement in higher plants.

1.3 Plastid division in higher plants

1.3.1 Observations of plastid division in higher plants

The replication of plastids in higher plants occurs at two specific stages of plastid development: the proplastid and the young chloroplast. Proplastid division provides a continuity of plastids between mitotic division of the plant cell so that a constant proplastid number is maintained in all post meristematic cells (Chaly, Possingham and Thomson, 1980). Proplastid division most frequently occurs either during or immediately prior to mitosis (Possingham, 1980; Whatley, 1986).

The replication of chloroplasts in the leaf mesophyll cells, and to a lesser extent in the epidermal cells and parenchyma sheath cells of the vascular bundle, facilitates the

accumulation by the leaf of the organelles responsible for maximal photosynthetic competence. Possingham and Saurer (1969) proposed that the majority of chloroplasts in the mesophyll cell arise from chloroplast division rather than the division of proplastid initials. Leech and Pyke (1988) also suggest that up to 90% of the chloroplasts of the mature wheat leaf mesophyll cell arise from the replication of young, green chloroplasts during post-mitotic cell expansion. The analysis of chloroplast populations has also suggested that all plastids within the cell are capable of division (Possingham, 1980; Leech and Pyke, 1988). A combination of chloroplast replication and chloroplast expansion provides a highly conserved chloroplast cover for the mesophyll cell.

The division of young green chloroplasts by the constriction of the plastid about a central isthmus followed by division into two daughter plastids has been observed and documented in several different species of higher plants (see Possingham and Lawrence, 1983; Whatley, 1988 and Boffey, 1992 for reviews). The initiation and completion of the chloroplast division process has also been observed in wheat *in vitro* (Leech, personal communication) although the complexity of the construction of higher plant leaves reduces the potential to view the process *in vivo*. A predicted sequence of events involved in the division of wheat mesophyll cell plastids was presented by Leech, Thomson and Platt-Aloia (1981) based on light and electron microscopical observations of frequencies and temporal co-ordination of the various plastid morphologies involved in plastid division. The interpretation of the exact order of the various phases in the process of plastid division varies between the species investigated (Leech, Thomson and Platt-Aloia, 1981; Whatley, 1982; Possingham and Lawrence, 1983; Leech and Pyke, 1988; Whatley, 1988); although whether this is due to differences between species or experimental conditions is not clear.

The study of the ultrastructural and morphological changes in dividing chloroplasts of wheat (*Triticum aestivum*) by Leech, Thomson and Platt-Aloia (1981) has enabled the chloroplast division process to be categorised into five stages. Chloroplast division begins with the constriction of the chloroplast about its equator into a dumb-bell-like appearance. The constricted plastid then becomes elongated along the plane of the major axis of the plastid. The chloroplast then assumes a 'peanut' shape with distortion at the ends of either

segment of the constricted plastid. The constricted, elongated plastid is then observed to rotate and fold about the constricted isthmus. Finally division is terminated by the fusion of the envelope membranes at the isthmus and separation of the chloroplast into two daughter plastids, each approximately half the size of the parent. Several observations of the morphology of the plastid during the division process are relevant to the investigation of division. The most notable observation is the appearance in several constricted plastids of deposits of electron opaque material at the isthmus of dividing chloroplasts in the transmission electron microscope. This material, occasionally observed as an annulus encircling the isthmus and termed the *plastid dividing ring* is suggested to be involved in the constriction of the isthmus (discussed in 1.3.3). The presence of, usually one or two, mitochondria at the isthmus of dividing chloroplasts in wheat has also frequently been observed (Chaly, Thomson and Possingham, 1980). The presence of mitochondria may be coincidental, but may indicate the requirement for energy resources by an active process involved in chloroplast division.

1.3.2 The initiation of chloroplast division in higher plants

The initiation of the chloroplast division process is characterised by the central constriction of the plastid into a 'dumb-bell' or 'hourglass' configuration. The stimulation of dumb-bell formation and the mechanisms controlling it are of considerable interest to the study of chloroplast division. Leech, Thomson and Platt-Aloia (1981) suggested that the formation of the central constriction follows a process comparable to the theoretical model of cytokinesis proposed by Greenspan (1977, 1978) which describes the spontaneous development of a centralised constriction in an oil droplet in response to variable surface tension. The Greenspan model was modified to relate to chloroplast division by Possingham and Lawrence (1983).

The model of cytokinesis proposed by Greenspan (1977) suggests that a reduction in the tension of the lipid membrane at the poles of the cell induces the movement of *tension forces* towards the equator. The resultant increase in membrane tension at the equator of the cell, increased by internal fluid movements stimulated by the change in tension, causes an invagination of the ovoid cell about the equator, equidistant from either pole. This invagination of the membrane may proceed almost to completion, but

Greenspan suggests that the forces of tension alone are not sufficient to facilitate the separation of the two constricted halves of the cell (Greenspan, 1978). The model adopted by Possingham and Lawrence proposes that chloroplast division is initiated by a reduction in the tension at the poles of the chloroplast causing an invagination of the plastid equator. The invagination is amplified by induced flow of the stroma within the plastid and proceeds to the development of a tight isthmus. Possingham and Lawrence predict an accumulation of membrane components at the isthmus which is followed by the separation of the two halves of the plastid into the two daughter chloroplasts. The accumulation of material at the constricted isthmus is likely to be the formation of the plastid dividing ring which is observed in dividing chloroplasts (Hashimoto and Possingham, 1989). It is likely therefore that, following the observations of Greenspan (1978), plastokinesis is finally achieved by the action of the plastid dividing ring rather than by the forces of tension alone. There is no proof that the initial constriction of the chloroplast is due to this effect; since there is presently no means of identifying the plastid dividing ring in intact chloroplasts, it is not possible to conclude whether the plastid dividing ring is present at the initiation of constriction or whether an initial invagination of the chloroplast is required to facilitate the association of the plastid dividing ring structure from its components.

1.3.3 The division of higher plant chloroplasts by central constriction facilitated by the plastid dividing ring

Once initiated the process of chloroplast constriction proceeds rapidly until division of the chloroplast into the two daughter plastids is achieved. Leech, Thomson and Platt-Aloia (1981) propose a time scale of approximately 20 minutes for the division of a single wheat chloroplast, derived from the relative proportion of chloroplast morphologies observed compared to calculated chloroplast doubling time. This rate of division may possibly be altered in other species whose chloroplasts are a different size to those of wheat; the mean chloroplast size of *Arabidopsis*, for example, is 10 fold larger than wheat chloroplasts.

The plastid dividing ring, suggested to be involved in plastid division (Leech, Thomson and Platt-Aloia, 1981) was demonstrated to exist as a complete ring wrapped

around the constricted isthmus of the dividing chloroplast by Oross and Possingham (1989). Electron micrographs of tissue sectioned transversely through the dividing isthmus, perpendicular to the major axis of the chloroplast showed the plastid dividing ring to be present as a single, or two concentric, rings associated with the envelope membranes. The localisation of the plastid dividing ring varies between species; in most lower plants studied, the ring is observed at the cytosolic face of the outer chloroplast envelope membrane. The plastid dividing ring is observed in wheat, oats, tobacco and spinach (Oross and Possingham, 1989) to be present as a *double* ring structure at both the cytoplasmic face of the outer envelope membrane and the stromal face of the inner envelope membrane.

The plastid dividing ring is initially observed at isthmi which have constricted to approximately a third of the plastid diameter, and is most easily resolvable in plastids where the isthmus has narrowed to a diameter of *c.*1μm (Hashimoto, 1993). Observations on dividing chloroplasts in oats (Oross and Possingham, 1989) demonstrate that the annulus of the plastid dividing ring proceeds to increase in width along the major axis of the dividing chloroplast as the isthmus constricts, often causing the isthmus to adopt a tubular structure along the major axis of the chloroplast as the plastid dividing ring expands. This behaviour of the plastid dividing ring suggests that it is composed of one or more filamentous structures of a defined length which tighten the isthmus by wrapping around the constriction, progressively overlapping itself by an increasing number of times. A filamentous plastid dividing ring would suggest that an actin/myosin structure was involved, however the chemical nature of the plastid dividing ring has not yet been confirmed in higher plants. Studies using immunocytochemistry to recognise actin have suggested that the plastid dividing ring in algae is comprised of, or includes actin (Hashimoto, 1992). However the techniques employed to confirm the activity of actin in the plastid dividing ring of lower plants have not yet been applied successfully to higher plants. Chloroplast constriction is often, however, accompanied by the presence of one or more mitochondria at the isthmus (Chaly, Possingham and Thomson, 1980), suggesting the action of an active, energy-requiring process, possibly supporting the role of an actin/myosin system.

1.4 Factors which affect chloroplast division in higher plants

The accumulation of the chloroplast complement in the photosynthetic cells of the leaf involves the replication of numerous young chloroplasts. Pulse-chase experimentation with dividing chloroplasts of spinach (Possingham, 1980) suggest that all chloroplasts within the mesophyll cell are capable of division during mesophyll cell development. Possingham, Hashimoto and Oross (1988) suggest that *c*0.5 x 10^7 chloroplasts are formed by division per leaf per day in expanding leaves of spinach and tobacco. Since chloroplast number is species specific, genetic control of this process of chloroplast accumulation would be predicted but no definite control mechanisms have as yet been recognised in higher plants. A large number of factors which affect chloroplast replication, either to stimulate or restrict the process, have been identified and their effects noted; however little evidence supports a mechanism for these effects. The effects of endogenous and exogenous factors on chloroplast division are considered in reviews by Butterfass (1979), Possingham, Hashimoto and Oross (1988) and, more recently, by Boffey (1992) and are discussed individually below.

1.4.1 The effect of mesophyll cell size on chloroplast replication

The initial observations suggesting that mesophyll cell chloroplasts increase in number during the expansion of the leaf initially implied a relationship between chloroplast replication and cell expansion (Possingham and Saurer, 1969). The close relationship between cell size and the accumulation of the chloroplast complement has been frequently observed in a number of species. Honda, Hongladarom-Honda, Kwanyuen and Wildman (1971) observed a close relationship between increasing mesophyll cell size and chloroplast number in spinach, sugar beet, chives and tobacco. Although the mean total chloroplast cover per cell was different for each individual species, it was observed in spinach that a constant proportion of the mesophyll cell was covered by the chloroplast complement during cell expansion. An association has also been observed in *Phaseolus vulgaris* (Whatley, 1977), wheat (*Triticum aestivum*) (Pyke and Leech, 1987) and more recently in *Arabidopsis* (Pyke and Leech, 1991). Chaly, Possingham and Thomson (1980) observed that, in spinach leaf discs cultured in light, an

80% increase in mesophyll cell size was accompanied by a 65% increase in chloroplast number. This effect was investigated in the wheat genus *Triticum* for a range of species, and also a range of cultivars of *Triticum aestivum* (Pyke and Leech, 1987), each of which demonstrated a tight relationship between chloroplast number and increasing mesophyll cell size.

The results of Ellis and Leech (1985) and Pyke and Leech (1987) suggest that, once plastid division is initiated, the mesophyll cell chloroplasts replicate until a maximum number is attained. Cell size is therefore a limiting factor to the replication and accumulation of the chloroplast complement, so that a constant chloroplast cover per mesophyll cell is maintained throughout mesophyll cell development. This is demonstrated to an extreme degree by the treatment of wheat leaves with maleic hydrazide which produces giant cells which may contain up to 600 chloroplasts (wild type mean of 150 chloroplasts) per cell. Furthermore, Leech and Pyke (1988) suggest that an initial increase in mesophyll cell size may be a stimulus for the initial division of chloroplasts in wheat.

The precise means by which the restriction of chloroplast number by cell size is caused has not been shown, but is discussed further in the discussion of chapter 4.

1.4.2 The effect of chloroplast size on chloroplast replication

Chloroplast expansion proceeds as a part of the development of young chloroplasts both before and after division (Possingham and Lawrence, 1983; Leech, 1984, 1986). Possingham and Lawrence suggest that during early cellular development, chloroplast size gradually increases so that successive divisions occur in increasingly larger chloroplasts. Ellis and Leech (1985) observed that the largest chloroplasts in the mesophyll cell were involved in division and are on average 1.5 fold larger than the mean size of non-dividing chloroplasts. Ellis and Leech suggest an optimum chloroplast size for division of *c.* 6μm^2 in wheat; an optimum size is also observed in *Arabidopsis* in this thesis (chapter 4).

An inverse relationship between chloroplast number and chloroplast size has been noted in the developing cells of several species. Possingham and Smith (1972) observe that in spinach leaf discs grown at low temperatures the chloroplasts are more numerous but of reduced size; at higher temperatures fewer larger chloroplasts are observed. A

similar effect has been noted more recently in the *arc* mutants of chloroplast division (Pyke and Leech, 1992, 1994). This effect may be misleading, suggesting a negative regulatory effect on chloroplast division by chloroplast size. However, analysis of *arc* mutant chloroplast phenotypes, discussed later, has suggested that the compensation of chloroplast number for size is an effect of reduced chloroplast division rather than increased chloroplast expansion. The importance of chloroplast size on the division process in higher plants is suggested to be considerable and is discussed in more detail in Chapter 4.

1.4.3 The effect of nuclear DNA on chloroplast replication

The nuclear DNA of the plant cell contains a high proportion of plastid genes, it would be logical to assume therefore that the nuclear DNA has a profound effect on the division and accumulation of the plastids. Approximately 70% of plastid polypeptides are coded for in the nucleus (Douce and Joyard, 1984), and the replication of the CtDNA is performed by an imported, cytoplasmically-synthesised polymerase (Possingham, Hashimoto and Oross, 1988). Inhibitors of the synthesis of 80s cytosolic ribosomes have a more pronounced effect on chloroplast number than inhibitors of chloroplast protein synthesis (Leonard and Rose, 1979). The control of the accumulation and replication of chloroplasts in *Arabidopsis* by nuclear genes has been illustrated by the isolation of nuclear *arc* mutants (Pyke and Leech, 1992, 1994) and is discussed in detail in 1.7. The nucleus evidently has a significant role in the genetic determination and control of chloroplast replication.

An alternative effect of the NcDNA on chloroplast replication is proposed by Butterfass, regarding the effect of nuclear DNA amount on chloroplast number. Butterfass (1979) suggests that the NcDNA affects plastid replication at two levels: firstly the duplication of the NcDNA at mitosis associated with proplastid division; secondly the effect of nuclear ploidy on chloroplast numbers in mesophyll cells.

The continuity of plastids during mitosis would logically require an association of proplastid division with nuclear division. Butterfass (1988) suggests that the replication of the NcDNA prior to cytokinesis would stimulate the replication of the proplastids, allowing for numbers of proplastids to be accurately apportioned to each of the newly

divided cells. Although such a proposed mechanism is plausible, proplastid division has been demonstrated to occur at various periods throughout mitotic cell divisions (Whatley 1986). The proplastids of cocoa (*Theobrama cacao*)(Whatley, 1986), for example, replicate in excess of mitotic divisions in the early cycles of cell division, after which the rate is reduced allowing for subsequent cell divisions to reduce the proplastid numbers to the appropriate levels. A duplication in cell ploidy has been observed to correlate with increased numbers of guard cell chloroplasts (proposed by Butterfass to equate to former proplastid numbers) which would suggest a relationship (Butterfass, 1979, 1983). No evidence from observations of proplastid division being synchronised with NcDNA replication is presented, however, which suggests that the alteration of nuclear DNA amount is not a direct stimulant for chloroplast replication.

The effect of NcDNA amount on chloroplast number has also been observed in polyploids, induced polyploids and anueploids (Butterfass, 1979, 1988). The increase in cell ploidy is always accompanied by an increase in chloroplast number per mesophyll cell (Butterfass, 1979, 1983). Butterfass (1983) suggests that the effect of nuclear DNA amount on chloroplast number is the primary controlling factor. Plastid size may also be affected by nuclear ploidy, for example sugar beet trisomics have been observed to display normal chloroplast numbers compared to the control, but increased chloroplast size (Butterfass, 1979). This observation, indicating an increased total chloroplast area per cell would require an increased cell size to occur, since the total proportion of the cell which is covered by the chloroplast complement is very consistent as the cell expands in many species. An increased total chloroplast area per cell may explain the effect of ploidy on chloroplast number. Pyke, Jellings and Leech (1990) observe a positive relationship between mesophyll cell size and ploidy in wheat. If one considers the tight correlation of chloroplast number with mesophyll cell size, discussed above, a more plausible effect of ploidy indirectly affecting chloroplast number by increasing mesophyll cell size may be considered. Furthermore, any effect of NcDNA amount would have to be considered as a restrictive effect, since chloroplast division may take place in the absence of NcDNA duplication or polyplody (Ellis, Jellings and Leech, 1983). Polyploidy may affect initial proplastid numbers per mesophyll cell; however even a doubling of proplastid numbers

would be of little significance to the eventual chloroplast population, 90% of which is produced from chloroplast division (Leech and Pyke, 1988). It is difficult to conceive a means by which the nuclear DNA may directly control chloroplast number by means of ploidy; it is more likely that the effect is only coincidental to chloroplast number due to its effect on other cellular factors.

1.4.4 The effect of chloroplast DNA on chloroplast replication

The theory of the endosymbiotic origin of plastids (Whatley and Whatley, 1981) suggests that the ancestors of plastids were originally independent phototrophic prokaryotes which developed subsequent symbiotic relationships with eukaryotic cells. It would therefore be logical to assume that these prokaryote ancestors were capable of autonomous replication prior to the symbiotic relationship. All plastids contain their own DNA, known as **CtDNA** or the ***plastome***, present in high copy numbers per plastid, between 310 and 810 copies per plastid in wheat (Boffey and Leech, 1982). The high degree of translocation of chloroplast genes to the nuclear DNA (NcDNA) suggest that genes for chloroplast division may be resident in the nuclear genome. However, the replication of CtDNA has frequently been observed to be associated with chloroplast division. The replication of the chloroplast genome is most frequently observed to precede chloroplast division (Boffey and Leech, 1982; Marrison and Leech, 1992). The plastome copies per plastid increase relative to chloroplast division during early development but then reduce as chloroplast division proceeds in the absence of CtDNA replication. This may suggest that CtDNA replication initiates the division of the chloroplast in which it resides, however studies of the effects of gamma irradiation, an inhibitor of CtDNA synthesis, show that chloroplast division may occur in the absence of CtDNA replication. The pale sectors of the barley mutant, *Albostrians* (Hashimoto and Possingham, 1989) contain undifferentiated proplastid initials, but similar CtDNA amounts to the adjacent wild type green sectors. The extremely high ploidy of the plastome makes it difficult to envisage that the increase of chloroplast DNA levels has a marked effect on chloroplast division.

Possingham and Lawrence (1983) suggest that the association of CtDNA replication and chloroplast division passes through three stages. Firstly, both cell division, plastid division, CtDNA replication and NcDNA replication occur at comparable rates so that during cell division, plastid numbers and plastome ploidy are constant per cell. Secondly, cell division gives way to cell expansion and CtDNA replication continues in the absence of NcDNA replication and chloroplast division. Thirdly, chloroplast division occurs, either in the absence of or in the final stages of CtDNA replication. The association between CtDNA replication and chloroplast division cannot be ignored, but it is unlikely that chloroplast division is stimulated by CtDNA replication. The effects on CtDNA amount of a lesion in chloroplast replication noted in the *arc* mutants are investigated in chapter 3.

1.4.5 The effect of light on chloroplast replication

The chloroplast's primary function is the harvesting of light energy. It would be reasonable to assume therefore that light may play a role in the control of the replication of chloroplasts. Light intensity and quality have been shown to affect chloroplast development and growth (Cran and Possingham, 1972; Leech, 1986), although plastid development is not necessarily light dependent (Kirk and Tilney-Bassett, 1978). Light has also been observed to induce the movement of chloroplasts in higher plants (Haupt, 1973; 1982), a similar effect on plastid constriction may therefore be envisaged.

Chaly, Possingham and Thomson (1980) observed an increase in chloroplast number of 65% in the cells of spinach leaf disks grown for five days in low intensity green light when transferred to high intensity white light. These preliminary experiments, however, did not consider the *energy* input from the light between samples. A repetition of the experiments with a balanced input of light energy between the treatments is required before these results may be verified. This increase was associated with an increase in cell size, so may not be a direct result of light stimulation on chloroplast division. However, 65% of the chloroplasts in the low light grown leaf disks demonstrated a constricted appearance, a number which subsequently reduced to 20% after 16 hours in the light indicating the stimulation of the completion of chloroplast division by the action of high intensity white light. Possingham (1973) observes that both red and blue laser light

stimulate chloroplast division in spinach leaf discs to a similar extent to white light. Green light and low intensity light, however, have no significant effect on chloroplast division.

The enhancement of chloroplast division by light is suggested by the observations of Butterfass (1979) that, in high intensity white light, chloroplast number increases with a relative decrease in chloroplast size, and *vice versa* for low intensity light, so that the total chloroplast cover remains constant. This effect may be the result of either an increase of chloroplast division due to high intensity light or a reduction in chloroplast expansion.

The stimulation of chloroplast division by light may seem plausible, but this may not apply to all plastids within the plant. Some plastids, such as the proplastids of the root or shoot meristems or the amyloplasts of the root are capable of division whilst receiving little or no light (Chaly, Possingham and Thomson, 1980). Possingham and Lawrence (1983) suggest that the photosynthetic activity of the chloroplast distinguishes it from other plastids in this respect, possibly requiring energy supplied by photosynthesis for division. The exact nature of this dependence is unclear; however intensity and quality of light must be accepted to have a significant effect on chloroplast division.

The cells of spinach leaf disks cultured in a 14 hour light : 10 hour dark environment are observed to display increased numbers of constricted chloroplasts during the first few hours of the light period (Chaly, Possingham and Thomson, 1980). This apparent stimulation of chloroplast division, however, does not correlate with an increase in plastid numbers, suggesting that the effect may be due to a reduction in the speed of constriction in the dark (Possingham, Hashimoto and Oross, 1988). The constriction of the chloroplast may therefore be reliant, to a degree, on photosynthetic products or the effects of phytochrome, but chloroplast division does not appear to follow a diurnal pattern.

1.4.6 The effect of energy supply and minerals on chloroplast replication

Possingham and Smith (1972) indicate that a reduction in sucrose in the incubation medium of spinach leaf disks causes a reduction in chloroplast number. The effect of a deficiency or excess of several vital minerals is also recorded. A deficiency of manganese, iron or nitrogen, vital components of chloroplast constituents, caused chlorosis of the leaves in spinach and a reduction on the numbers of chloroplasts per mesophyll cell

(Possingham, Hashimoto and Oross, 1988). This deficiency also reduces cell size, however, which may be the cause of the decrease in chloroplast number. An increase in calcium levels also reduces chloroplast numbers (Possingham, Hashimoto and Oross, 1988). Conversely, increases in potassium and sodium have been observed to increase mesophyll cell size and, possibly as a result of this change, chloroplast number. A mechanism for the effect on chloroplast number of these several nutritional effects is unclear, although they may feasibly be secondary effects of perturbations to other processes.

1.4.7 The effect of temperature on chloroplast replication

The incubation of spinach leaf disks at temperatures varying between 12 and 35°C has a marked effect on chloroplast number and size. Leaf disks incubated at low temperature contain fewer larger chloroplasts than the control, whilst those incubated at higher temperatures contain more, smaller chloroplasts (Possingham and Smith, 1972). A plausible explanation for this effect may be that the initiation of chloroplast divisions is retarded at low temperatures and stimulated at high temperatures. The relationship between plastid number and plastid size has been observed in several instances (discussed above), but so far suggests that changes in plastid size act to compensate alterations in plastid number per cell. A direct alteration to plastid division is therefore implied by these observations.

1.5 The genetic co-ordination of chloroplast division in higher plants

The study of the control of chloroplast division in higher plants has concentrated to date on the effect of several physical factors on the development of the chloroplast complement. The plastids of the higher plant cell, however, are very closely associated with the cell both physically and genetically. The proposed origin of the chloroplast as an initially independent endosymbiotic prokaryote (Whatley and Whatley, 1981) implies that the chloroplast ancestor was capable of reproduction independently of the host cell nucleus. The genes controlling the division of the chloroplast are therefore likely to have been retained in either the chloroplast or nuclear DNA of the plant cell. The analysis of the

nuclear DNA would be predicted to reveal genes which affect or control the division and accumulation of the chloroplast in higher plants.

The analysis of the genes controlling chloroplast division would require the use of a species of higher plant which has a chloroplast complement that is typical of the majority of angiosperms. The species should also be amenable to the requirements of genetic analysis, giving a real possibility of the isolation of relevant nuclear loci by molecular biological techniques. The plant chosen as the model for the project was the ruderal crucifer *Arabidopsis thaliana* which has become the model plant for genetic analysis in recent years (Bowman, Yanofsky and Meyerowitz, 1988)

1.6 *Arabidopsis thaliana*, a model system for the analysis of the genetic control of chloroplast division in higher plants

Within the past decade *Arabidopsis thaliana* has become the model plant for the study of plant metabolism and morphogenesis by the isolation and characterisation of relevant genes by molecular biological techniques (Bowman, Yanofsky and Meyerowitz, 1988; Dean 1993; Pyke 1994). The isolation and characterisation of large numbers of plant genes has been facilitated by the use of the *Arabidopsis* model system (for a review of the range of mutants of morphogenesis in *Arabidopsis* see Pyke, 1994) and a large proportion of the advances in plant molecular biology have come about as a result of the use of this plant. *Arabidopsis thaliana* is an ideal system for the isolation of higher plant genes due to several characteristics:

(i) The *Arabidopsis* plant itself is small, may be grown in large numbers with relative ease and has a very short generation time, one generation from seed to seed is usually 6 to 8 weeks. The plant may be grown in glasshouse conditions to yield up to 10,000 seeds per plant or may be cultivated in tissue culture or sterile conditions, also with considerable yield of seed. *Arabidopsis* is normally cleistogamous, allowing for the reliable accumulation of inbreeding strains; but may be outcrossed by dissection of the immature floral bud followed by manual pollination should hybrid crosses be required.

(ii) The genome of *Arabidopsis* is considerably smaller than most studied plant systems, with a haploid genome size of approximately 100Mb DNA (Pruitt and

Meyerowitz, 1986) packaged between five chromosomes. The DNA of *Arabidopsis* contains less untranslated and repeat DNA than any higher plant studied (Bowman, Yanofsky and Meyerowitz, 1988), thus increasing the efficiency of studying the active sequences of the *Arabidopsis* genome.

(iii) Transformation of *Arabidopsis via* the *Agrobacterium tumefaciens* system is now routine in several laboratories. *Agrobacterium tumefaciens* infection may be performed by root tissue infection (Valvekens, van Montagu and van Lijsebettens, 1988), although the resultant growth by tissue culture may result in a high degree of somaclonal variation (Marks and Feldmann, 1986). Alternative methods for transformation avoiding tissue culture include the use of seed infection (Feldmann and Marks, 1987) or an *in planta* vacuum infiltration of intact tissue (Sangwan, Velu, Cobanov, *et al*, 1993).

(iv) The characterisation of the *Arabidopsis* genome has become the subject of a co-ordinated international programme. The *Arabidopsis* physical and genetic maps have been described in considerable detail, and are being constantly updated. The physical map of *Arabidopsis* presently contains over 120 visible morphogenetic markers distributed across the five chromosomes (Dean, 1993) and is constantly being increased as more researchers localise characteristic mutant phenotypes to the genome, each increasing the accuracy of the physical map. The accurate localisation of a mutant locus by recombination of the mutation with a locus revealed by a Restriction Fragment Length Polymorphism (RFLP) is greatly facilitated by a very high concentration of mapped RFLP markers in *Arabidopsis* (Dean, 1993). The recent development of RFLP mapping sets such as Random Amplified Polymorphic Sequences (RAPD; Reiter, Williams, Feldmann, *et al*, 1992); *Arabidopsis* RFLP Mapping Set (ARMS; Fabri and Schäffner, 1994) and Co-Amplified Polymorphic Sequences (CAPS; Konieczny and Ausubel, 1993) has greatly facilitated the rapid localisation of a mutant locus to one arm of one of the five chromosomes. The construction of several populations of *Arabidopsis* mutagenised by insertional mutagens such as T-DNA (Feldmann and Marks, 1987) and transposons (Bancroft, Bhatt, Sjodin *et al*, 1992; Dean, Sjodin, Page, Jones and Lister, 1992) is also anticipated to facilitate the rapid isolation of several loci by the generation of mutant loci which are tagged with a DNA fragment of known sequence.

(v) The construction of Recombinant Inbred lines (Reiter, Williams, Feldmann, *et al*, 1992; Lister and Dean, 1993) with which the various genetic markers developed for *Arabidopsis* may be accurately localised to the physical map (Jarvis, Lister and Dean, 1994) has enabled an accurate co-ordination of the genetic and physical maps, such as was initiated by Haage, Hanley, Cartinhour *et al* (1993).

(vi) Finally, and potentially of most value, the characterisation of the *Arabidopsis* genome into contiguous regions of Yeast Artificial Chromosome (YAC) and cosmid clones of genomic DNA will facilitate the rapid isolation of gene sequences of mapped loci. The identification of each area of the *Arabidopsis* genome as a single or small number of catalogued genomic clones should remove the requirement for long-term programmes of chromosome walking for the isolation of loci. The construction of YAC contigs is presently a co-ordinated project between the research groups of H. Goodman (chromosomes 1, 2 and 3) and C. Dean (chromosomes 4 and 5) (Hwang, Kohchi, Hauge, *et al*, 1991; Schmidt, Cnops, Bancroft and Dean, 1992; Dean, 1993; Schmidt, West, Love, *et al*, 1995).

1.7 The isolation of *arc* mutants of chloroplast number and size in *Arabidopsis*

The use of mutagenesis to study the action of genes has been widespread for several years and has enabled the isolation and characterisation of several loci. The disruption of a biological process as a tool to study its mode of action is not a new concept and the use of mutants deficient in particular genetically controlled processes has been used since Mendel's experiments. The use of mutagenesis as a tool to study morphogenesis and development in *Arabidopsis* has recently been reviewed substantially by Pyke (1994) and the range, variety and potential of this approach is clearly illustrated in Bowman (1993).

The study of the genetic control of chloroplast division in higher plants by the use of mutagenesis was initially approached by Pyke and Leech (1991) who devised a screen of EMS mutagenised individuals of *Arabidopsis* to detect mutant chloroplast phenotypes. The initial screen of the mutagenised population was carried out semi-automatically with

the assistance of an image analysis system (Pyke and Leech 1991). In the screen the criteria for a mutant phenotype was a plant which displayed a significantly different chloroplast number per mesophyll cell to wild type (Landsberg *erecta*). A sub-cellular screen was chosen rather than a screen of whole plant phenotypes since the accuracy of a cellular analysis for the chloroplast phenotype was correctly anticipated to be the more reliable. A number of putative mutants were isolated which displayed either more or fewer chloroplasts per mesophyll cell than wild type. The mutants, termed ***arc*** mutants (***a***ccumulation and ***r***eplication of ***c***hloroplasts), were stable over at least 8 generations and, through backcrossing to wild type, demonstrated to be nuclear recessive (Pyke and Leech, 1992, 1994). The five *arc* mutants characterised from the EMS screen either display a mutant chloroplast phenotype of a few, large chloroplasts or of more smaller chloroplasts than wild type. The accumulation of chloroplast number, if it occurs at all, is very closely associated with increase in mesophyll cell size, which is also observed in wild type *Arabidopsis*. The compensation of chloroplast size for chloroplast number producing few large or many small chloroplasts is also consistent between mutants so that in all phenotypes a constant total chloroplast area per mesophyll cell which is comparable to wild type is maintained during cell development.

The six mutants characterised from the EMS screen represent separate mutations at five independent nuclear loci. Mutants *arc*1, *arc*2 and *arc*3 are described in Pyke and Leech (1992); *arc*5 is described, with further data on *arc*1, *arc*2 and *arc*3, in Pyke and Leech (1994); details of *arc*4 are unpublished. The characteristics of the *arc* mutants are summarised in Figure A at the beginning of this thesis.

***arc*1** mesophyll cells have more, smaller chloroplasts than wild type. The mean mesophyll cell size in *arc*1 is approximately half that of Landsberg *erecta* wild type, such that the density of chloroplasts per unit of cell area in *arc*1 is twice that of wild type. The mutant phenotype is consistent in all *arc*1 chloroplast-bearing cells except the parenchyma sheath cells where the *arc*1 mutant effect is absent. The nature of this tissue specificity is not clear. The *arc*1 mutant is slightly pale in early seedling development but becomes greener during subsequent growth so that by *c*.28 days it does not differ from wild type.

arc2 displays a mesophyll cell phenotype of slightly fewer chloroplasts than wild type which are of variable size ranging up to three fold larger than Ler wild type.

arc3 mesophyll cell chloroplast number does not increase during cellular development, suggesting that chloroplast division does not normally occur in this mutant. The chloroplasts of *arc*3 are approximately six fold larger than Ler wild type and are frequently irregular in shape. Whether this irregular shape is a function of the mutation or an effect of the large chloroplast size is unknown. Two mutant alleles of the *ARC*3 locus have been isolated, *arc*3-1 and *arc*3-2; however they do not display significantly different mutant phenotypes. The analysis of the *arc*3 phenotype has been carried out on *arc*3-1.

arc4 displays a similar, though more moderate chloroplast phenotype to *arc*2. The size of *arc*4 chloroplasts varies from wild type, although a restricted distribution of plastid sizes is observed compared to *arc*2 or *arc*3.

arc5 is one of the most interesting *arc* mutants isolated to date. The *arc*5 mesophyll cell chloroplasts are similar in number and size to *arc*3, suggesting that chloroplast division does not normally occur. However the majority of *arc*5 chloroplasts display a degree of constriction about the equator of the chloroplast, reminiscent of a dividing chloroplast. The investigation of this phenotype suggests that the *arc*5 mutation somehow arrests division of the plastid midway through the process so that constriction is not completed and the chloroplast, trapped in this relic of division, subsequently expands in parallel to cell expansion. *arc*5 is therefore a very valuable resource for the analysis of the factors determining chloroplast division.

The construction of double mutants between *arc*1 and both *arc*3 and *arc*5 which are homozygous recessive for both mutant alleles and display the two *arc* mutant phenotypes simultaneously has indicated that the *arc*3 and *arc*5 alleles do not completely inhibit chloroplast division. The *arc*1/*arc*3 and *arc*1/*arc*5 double mutants both display increased numbers of chloroplasts per mesophyll cell compared to the *arc*3 and *arc*5 parents, however neither display a wild type chloroplast number. The *arc*1/*arc*3 double mutant has slightly more chloroplasts than *arc*3; the *arc*1/*arc*5 double mutant has significantly more chloroplasts than the *arc*5 mutant, with a large proportion of division profiles evident. The analysis of double mutants has suggested that *arc*3 is a lesion in the initiation of division,

*arc*5 in the completion of the process. However neither mutant is a null effect, so that when placed in a background where division is increased by some means, such as in *arc*1, chloroplast division may proceed at a moderate rate.

Despite quite radical perturbations in the construction of the chloroplast complement in the *arc* mutants, several consistent traits were noted. The increase in chloroplast number (with the exception of *arc*3 and *arc*5) and the increase in total chloroplast area were closely associated with increasing mesophyll cell size. The smallest, post-mitotic cells of both the wild type and all *arc* mutants and double mutants contained approximately 14 chloroplasts per cell. This number is suggested to be the mean number of proplastids apportioned to each cell by the end of meristematic activity, indicating that all *arc* mutants are mutants of *chloroplast* division rather than proplastid division. The most interesting observations, however, were that the plants of each *arc* mutant displayed no significantly deleterious mutant phenotype. The *arc*1 mutant is slightly pale in its early development, as are the *arc*1/*arc*3 and *arc*1/*arc*5 double mutants, however the *arc*1 mutant seedling becomes greener after approximately 25 days post germination. The *arc* mutants generally are very slightly slower to develop than wild type, the first leaf of the mutants is fully expanded by approximately 2 days after Ler wild type. The effect of the *arc* mutations on the fitness of the *Arabidopsis* plant in natural conditions may be more extreme than in controlled plant growth conditions, but the apparent normalcy of the *arc* mutant plant phenotypes is rather surprising considering the radical alterations which have occurred to the supposedly vital process of chloroplast accumulation.

An ultrastructural analysis of the internal structure of the *arc* mutant chloroplasts by Dr E. Robertson (unpublished) has revealed a surprisingly normal thylakoid structure in all *arc* mutants except *arc*5. The development of the thylakoid system is apparently unperturbed by the lack of division of the chloroplast. The *arc*5 mutant displays a high degree of contortion of the thylakoid membrane system indicative of a dividing chloroplast (Robertson, Rutherford, Pyke and Leech, in preparation; discussed further in chapter 4), but the construction of the thylakoid membrane and granal stacking in *arc*5 is not significantly perturbed. My contribution to the analysis of *arc*5 is described in chapter 4.

The high degree of diversity of the *arc* mutants displays the complexity of the genetic control of chloroplast division in higher plants. The apparent lack of significantly deleterious effects of the *arc* mutations with very few chloroplasts also questions the necessity of chloroplast division in *Arabidopsis*. The isolation and characterisation of the *ARC* genes whose disruption causes the several *arc* phenotypes described is anticipated to reveal a better understanding of the nature of these mutant effects and their significance to the mesophyll cell.

1.8 Summary of the experimental approach

The EMS mutagenised *arc* mutants are of significant value to the analysis of chloroplast division in *Arabidopsis*, they are not, however, ideal for the isolation of *ARC* genes. The EMS mutagen commonly induces mutation by single base alterations, which would require a map-based cloning strategy to isolate the mutagenised gene locus (Redei,1992). Several *Arabidopsis* genes have been isolated by this method of cloning, however the strategy is a long-term and labour-intensive approach. A more efficient approach involves the use of gene tagging, the mutagenesis of a locus by the insertion of a DNA fragment of known sequence which may subsequently be identified along with the mutagenised locus. Two systems for gene tagging in *Arabidopsis* are presently being developed: the use of the T-DNA from the *Ti* plasmid of *Agrobacterium tumefaciens* and the use of transposons such as the *Ac/Ds* system of maize. Two populations of seeds from insertionally mutagenised populations were kindly donated by Dr K. Feldmann (T-DNA mutagenised) and Dr C.Dean and co-workers (transposon mutagenised).

The primary aim of the work described in this thesis was to isolate tagged *arc* mutants from T-DNA or transposon mutagenised populations which would facilitate the isolation of the several *ARC* loci in *Arabidopsis*. The isolation of *arc* mutants from these populations is described in chapter 3. The tagged mutants, once isolated, would be characterised phenotypically and tested for allelism to existing *arc* mutants. It was anticipated that tagging of the mutant locus would be demonstrated by the cosegregation of the mutant phenotype with a marker to the T-DNA or transposon after a backcross of the mutant individual to wild type. The presence of the marker in all mutant siblings of the F_3

generation of the backcross would indicate that the mutagen was localised at the mutated locus. A segregation of mutant F_3 siblings which did or did not contain the marker would suggest that either the mutagen was located near to or was unassociated with the mutant locus, and that an alternative mutagen was responsible for the *arc* phenotype. Only those mutants in which it could be demonstrated that the T-DNA or transposon was located within the *ARC* locus would be of real value for the isolation of the *ARC* gene. The known sequence of the T-DNA or transposon would then be used to retrieve fragments of the *ARC* DNA flanking the mutagen. The sequence of the isolated *ARC* gene fragments could then be used to probe for homology in cDNA or genomic libraries of *Arabidopsis* DNA, potentially leading to the isolation of clones constituting the complete *ARC* gene sequence. The analysis of the gene sequence was anticipated to lead to suggestions of the *ARC* gene product and its precise role in the development of the mesophyll cell chloroplast complement.

Associated with the analysis and isolation of tagged *arc* mutants, the analysis of the physical controls to chloroplast division was approached by the comparison of the *arc*5 mutant (which apparently halts the latter stages of chloroplast division) with Landsberg *erecta* wild type, described in chapter 4. It was anticipated that the late onset of the *arc*5 mutant effect would facilitate the analysis of the frequency, spatial and temporal distribution and limitations to the chloroplast division process in *Arabidopsis*. This analysis was anticipated to clarify the mechanism by which the mesophyll cell controls the accumulation and development of the chloroplast complement in higher plants.

The characterisation of the phenotype of the most extreme *arc* mutant, *arc*6, is described in chapter 5. *arc*6 mesophyll cells may contain as few as a single chloroplast, suggesting that both chloroplast and proplastid division are perturbed by the *arc*6 mutation. The genetic locus of the un-tagged *ARC*6 gene was determined by RFLP mapping, to be situated on chromosome 5, between two RFLP markers, m247 and DFR.

The only tagged mutant, *arc*11 was used to isolate the first *ARC* gene sequences in higher plants, described in chapter 6. The transposon tagged nature of the *arc*11 mutant enabled the plant DNA sequences flanking the transposon to be isolated, cloned and sequenced. The nucleotide sequence of a fraction of the *ARC*11 gene is anticipated to be

of considerable value for the isolation of cDNA or genomic clones of *Arabidopsis* DNA which contain the entire *ARC*11 gene.

The comparison of *arc* mutant phenotypes and their interaction with the cell and with each other has facilitated the development of several theories concerning the control of chloroplast division in *Arabidopsis*, which are summarised in chapter 7. Further research may reveal the degree to which these observations and suggestions may be applied to species other than *Arabidopsis*, leading to a clearer understanding of the control of chloroplast division and accumulation in higher plants.

CHAPTER 2

Materials And Methods

Details of the origin and composition of the chemicals and biological products mentioned below are included in Appendix A

2.1 Growth and harvesting of *Arabidopsis* plant tissue

2.1.1 Origin of the plant material

The Landsberg *erecta* (Ler) wild type *Arabidopsis* seeds were donated to this laboratory by Dr C. Dean. The Wassilewskija (WS) wild type seed was donated by Dr K. Feldmann, *via* the Nottingham *Arabidopsis* Stock Centre (NASC) (stock number N1601). Columbia (Col) wild type seed was also obtained from the NASC (stock number N933).

An EMS mutagenised population of Landsberg *erecta* ecotype seeds was obtained from Lehle Seeds, Arizona, USA. The T-DNA mutagenised lines of WS ecotype were obtained (by permission of Dr K. Feldmann, University of Arizona, USA) from NASC (stock number N3115) in 49 pools of 100 mutagenised lines each. The *Nae*I-deleted stable *Ac*(Δ*Nae*I *Ac*) transposon mutagenised seed was donated by T. Page and C. Dean, Cambridge Lab, John Innes Centre, Norwich, UK. The seeds were taken from progeny of four individual transformants (02213-3, 02213-10, 0383-3 and 0383-8) each of which was transformed with a single *Ac*-bearing T-DNA (section 3.2.8). Approximately 12 seeds were taken from each of 680 lines and each line stored separately.

2.1.2 Growth and harvesting of Arabidopsis *plants on soil*

The *Arabidopsis* seeds were sown on 350x210 mm seed trays filled with Fisons Leavington F2 compost. Prior to sowing, the compost was gently pressed flat then thoroughly soaked with water and left to drain for at least one hour. Seeds were sown thinly through a disposable plastic Gilson P1000 micropipette tip. Alternatively, seeds could be placed individually onto the soil with the moistened tip of a cocktail stick. Seeds sown for the EMS and T-DNA mutant screens were sown in 96-square wire grids placed on top of the compost, one seed per square. The individual plants could then be identified by tray and grid square number. Once sown, the seeds were covered with a thin layer of compost from a fine sieve. The seed trays were covered with a clear plastic lid and left unwatered until two days post-germination. Plants were grown in controlled environment

growth chambers at 20°C (with a 5°C night depression), 70% relative humidity and a light intensity of 60 Wm^{-2}

The mature *Arabidopsis* plants set seed under growth room conditions after approximately 8 weeks. Once the bolts and siliques were dry, the floral bolts were cut away from the plant and teased gently with the fingers over a sheet of paper. The dry siliques dispersed their seeds easily and the collected seed was then sieved, to remove the chaff of siliques and septa, and stored. If seed was required from individual plants within one tray, a 80mm x 200mm clear plastic bag was placed over the newly bolted plant. The bolts developed and set seed within the plastic bag; upon harvesting the individual plants, the base of the bolts were cut and the bag inverted to collect seed without danger of contamination. Individual siliques from outcrosses between *Arabidopsis* plants were harvested with forceps and stored individually in Eppendorf microfuge tubes.

Sieved seed was stored in Eppendorf tubes with a pierced lid placed in a sealed container containing silica gel to desiccate the seed. Seeds were stored at 4°C. Freshly harvested seed was observed to germinate best if kept in these conditions for at least 48 hours prior to sowing.

2.1.3 Growth of Arabidopsis *seedlings on nutrient medium*

Growth medium plates were prepared as detailed in Valvekens, Van Montagu and Van Lijsebettens (1988) using 1.2% Difco Bacto agar rather than 0.8%. The 1.2% agar was added to the nutrient mix and autoclaved (10 minutes purge, 20 minutes sterilisation (121°C), 20 minutes cooling). Sterile growth medium was allowed to cool to 55°C before pouring approximately 20ml into sterile 90mm diameter plastic petri dishes in a laminar flow hood. Growth medium plates were allowed to set and could be stored at 4°C for up to 1 month before being sown with sterile *Arabidopsis* seed.

The seed sterilisation procedure adopted was as detailed in Balcells (1992). The seed sterilisation, washing and sowing procedures were carried out in a laminar flow hood. Seeds to be sterilised were placed within a quarter-folded 55mm diameter Number 1 Whatman filter paper. The open end of the filterpaper sachet was folded shut, sealed with a plastic paper clip and labelled in pencil. The sachet was then gradually immersed in 70%

Ethanol and soaked for 2 minutes, followed by immersion in 5% sodium hypochlorite + 0.5% SDS for 15 minutes. From this point onwards the use of sterile equipment and conditions was essential. The sachets were rinsed three times in sterile double-distilled water for 5 minutes each. The sterile sachet of seeds was opened out in a sterile petri dish and the seeds were transferred individually to the growth medium with a sterile cocktail stick. Vernalisation of the seeds by placing the plates in the dark at 4°C for 48 hours produced uniform germination of seeds upon removal to growth room conditions.

Sterilised seeds to be assayed for selection of kanamycin resistance were sown on sterile 1.2% agar nutrient medium containing 50mg/l kanamycin. Kanamycin was prepared in stock solutions of 50mg/ml, filter sterilised and aliquotted for storage (kanamycin stock solutions could be stored for up to 6 months at -20°C). Kanamycin was added to the sterilised growth medium when the medium was cooled to approximately 55°C to avoid heat inactivation of the antibiotic.

The criteria for kanamycin resistance in *Arabidopsis* seedlings was the growth of the first leaves. Kanamycin sensitive seedlings could germinate and produce cotyledons on kanamycin medium, but were inhibited from further growth and were bleached and chlorotic in appearance. It was also noted that the root growth of kanS seedlings was retarded compared to kanR seedlings.

2.2 Outcrossing of *Arabidopsis* plants

2.2.1 Outcrossing procedure for Arabidopsis

Arabidopsis thaliana is a cleistogamous species with self-fertilisation occurring immediately prior to the opening of the floral bud. Therefore, for outcrossing between individuals, the unopened, immature buds of the plant were dissected prior to self fertilisation. Bolts were chosen for crossing when approximately 50mm high with a newly formed inflorescence of unopened buds. The younger axial buds from lower down the length of the bolt were removed carefully with a pair of ultrafine forceps. Approximately five of the largest (ideally 2mm long) buds were chosen for dissecting. Those buds which had opened sufficiently for the edges of the white petals to be visible were discarded since in these more mature buds self fertilisation was likely to have occurred already . The buds

not selected for the cross were removed using forceps, leaving the selected buds intact. To avoid contamination of the stigma whilst the buds were dissected, the fingers of both hands were washed with ethanol to remove any pollen grains on the skin, also the forceps were washed with ethanol and dried before dissection of the bud. The green sepals, white petals and immature anthers were removed from the bud, leaving only the stigma. Care was taken not to damage the stigma, inflorescence pedicel or bolt during dissection. At least five mature flowers were taken individually from plants of the second genotype to be used in the cross and their pollen-laden anthers brushed several times against the exposed stigma of the dissected bud. The pollinated stigma was then covered with a protective hood made from clingfilm to prevent any further pollination by neighbouring plants. The clingfilm hood was removed from the dissected buds after fertilisation had occurred and the stigmas had begun to darken and lengthen, approximately three days after the cross was performed. The resultant individual siliques from the crosses were allowed to develop and dry to a light brown colour before harvesting individually in Eppendorf microfuge tubes. The siliques were kept in desiccating conditions at 4°C for seven days before sowing for increased efficiency of germination. The seed from individual siliques was sown separately so that siliques which had accidentally self fertilised could be identified when the leaf tissue was analysed for its *arc* phenotype.

2.3 Preparation of *Arabidopsis* leaf material for microscopical examination

2.3.1 Preparation of Arabidopsis *leaf material for maceration into a cell suspension*

The protocol for the fixation and preparation of *Arabidopsis* leaf material for the visualisation of isolated cells follows Pyke and Leech (1987), adapted from Possingham and Smith (1972).

Arabidopsis leaf material (usually the first leaf) was cut from the plant with a new double-edged razor blade and fixed in 3.5% (v/v) glutaraldehyde in the dark for one hour. The glutaraldehyde was replaced by 0.1M Na_2EDTA (pH 9) and the sample heated at 60°C for 2 hours. The leaf material separated best if the sample was then placed at 4°C

overnight before maceration. The leaf material was stored at 4°C in the dark for up to 12 months without significant deterioration in the quality of the tissue.

A small section of the Na_2EDTA-treated leaf was removed to a glass microscope slide and teased gently apart in a drop of Na_2EDTA with the blunt end of a scalpel handle before covering with a Number 1 coverslip for viewing with Nomarski differential interference optics on a Nikon Optiphot microscope (Pyke and Leech, 1987) linked to an image analysis system (Seescan Imaging Ltd, Cambridge, UK).

Leaf tissue where the individual cells were to be stained for starch was macerated in Lugol's iodine (6% (w/v) KI and 4% (w/v) iodine, diluted 4 fold before use).

2.3.2 Fixation, embedding and sectioning of Arabidopsis *leaves*

This procedure follows the protocol of Marrison and Leech (1992). The *Arabidopsis* leaf sample was removed using a double edged razor blade; the petiole was retained for ease of orientation of the sample in the subsequent embedding procedure. Samples were fixed individually in glass vials overnight at room temperature in the dark. The fixation solution consisted of 3% (w/v) paraformaldehyde, 50% (v/v) ethanol, 5% (v/v) acetic acid. The paraformaldehyde was stirred in 22.5ml double distilled water at 60°C for 30 minutes, 4 drops of 5M NaOH were added (the paraformaldehyde will not dissole without his) and the solution was allowed to cool before the addition of the ethanol and acetic acid to make a final volume of 50ml.

After fixation, the leaf material was dehydrated through an ethanol series. The fixative was removed with a disposable pasteur pipette and replaced with 75% ethanol for 15 minutes, followed by 85% ethanol for 15 minutes, 4 changes of 95% ethanol for 15 minutes each, then 4 changes of 100% ethanol for 15 minutes each.

For the Polyethylene glycol 1500 (PEG) infiltration of the tissue and PEG embedding, PEG was liquified by gentle melting in a 56°C waterbath. Solutions of 20% (v/v), 50% (v/v), and 70% (v/v) PEG in absolute ethanol were prepared and kept at 56°C. 20% PEG was slowly added to the sample until approximately 10% PEG was achieved in the vial. Samples were rotated in this solution for 30 minutes. Subsequent stages in the PEG infiltration procedure were undertaken in a non-shaking waterbath at 56°C. The 10% PEG was removed and replaced with 20% PEG, 50% PEG and 70% PEG for 30 minutes

each, 100% PEG for 1 hour, 100% PEG overnight and a second 100% PEG overnight, all at 56°C. Care was taken to avoid any contamination of the PEG solutions with water. The samples were arranged using a blunt cocktail stick in plastic peel-away moulds containing fresh molten PEG. Unsectioned blocks were stored at room temperature and sectioned blocks were stored at 4°C.

2.3.3 Sectioning of PEG-embedded material

The excess PEG was cut away from around the sample using a scalpel, leaving a block of approximately 10mm x 4mm, with the 4mm edges cut further to produce a trapezium face for the block face. The sample block was then fixed to a wooden base using melted PEG and allowed to set well at 4°C. 5 or 7µm sections were cut with a stainless steel blade on the Spencer AO 820 microtome. The sections ribboned well when the blocks were stored at 4°C, so that several serial sections could be placed on one slide. Sections were adhered to Poly-L-Lys coated slides (Robertson and Leech, 1995). Sections were placed flat on the water-moistened slide and dried down on a heating block at 40°C overnight.

2.3.4 Staining of DNA in sections with DAPI and visualisation of fluorescence under ultraviolet illumination.

DAPI was prepared in stock solutions of 0.1mg/ml in water and stored at -20°C. 10µl of DAPI stock was mixed with 990µl Citifluor mountant and added to the section before addition of a number 1 coverslip, sealed at the edges with nail varnish, before observation. DAPI fluorescence was visualised by excitation of the tissue under UV light (excitation filter 365nm, 400nm barrier filter) on a Nikon Microphot FXA microscope. DAPI stained sections were photographed with Kodak Ektachrome 400 colour slide film on automatic exposure in a Nikon UFX camera.

2.3.5 Fixation and embedding of Arabidopsis *tissue in Spurr's epoxy resin for ultrastructural studies*

The fixation and embedding of leaf tissue for the analysis of cellular structure in section was carried out with Spurr's epoxy resin (Spurr, 1969), following the protocol outlined in Jellings and Leech (1984)

The leaf material was cut into 2-3mm sections using a double-edged razor blade before fixation and immediately placed in glass vials with tight-sealing plastic lids, containing 2.5% (v/v) gluteraldehyde (E.M. grade) fixative for 1 hour at room temperature. The samples were washed for 3 x 10 minutes rotating in 0.1M phosphate buffer (6mM KH_2PO_4, 14mM KH_2PO_4, pH 7.2 in double distilled water). The buffer was then removed and replaced with sufficient 1% (v/v) osmium tetroxide (diluted in 0.1M phosphate buffer) to cover the samples and rotated for 2 hours at room temperature, then washed for 3 x 10 minutes in 0.1M phosphate buffer. The samples were then dehydrated through an acetone series of 10% (v/v) with double distilled water, 25%, 50%, 70%, 90%, 100% (all v/v) for 15 minutes each. This was then followed by two further changes of 100% acetone for 30 minutes each. Samples could then be left at 4°C overnight if necessary. Spurr's resin was prepared fresh, consisting of 15g Vinylcyclohexene dioxide (VCD), 9g Diglycidyl ether of polypropyleneglycol (DER736), 39g Nonenyl succinic anhydride (NSA) and 0.4g Dimethylaminoethanol (DAE). Whilst the samples were rotating, 3 drops of Spurr's resin were added every 20 minutes for 2 hours using a disposable pasteur pipette. Half of the sample volume was then removed and 5 drops added every 20 minutes for 2 hours; half of the mixture was again removed and 5 drops of Spurr's resin added every 20 minutes for 2 hours. The whole mixture was then removed and replaced with Spurr's resin for 1 hour, and repeated to be left overnight. The samples were then embedded in Spurr's resin in rubber moulds and polymerised overnight at 72°C in an oven.

2.3.6 Sectioning and staining of Spurr's embedded material for visualisation with the light microscope

Spurr's resin blocks were trimmed using a razor blade and 1μm thick sections were cut on a Reichert microtome. Sections were cut with a glass knife and floated onto a double distilled water reservoir at the back of the knife. The sections were removed to a drop of water on a microscope slide and dried down until the water had completely evaporated. Sections were stained with toludine blue and heated for approximately 25 seconds on a 60°C hot plate.

2.3.7 Sectioning and staining of Spurr's resin-embedded material for visualisation with the Transmission Electron Microscope

Ultrathin sections of Spurr's resin embedded material were cut on an LKB Ultratome III microtome using a diamond knife and mounted on 200-square copper grids coated with 0.3% Formvar. Double staining in uranyl acetate and lead citrate preceded examination on a Jeol JEM-1200EX electron microscope. The transmission electron microscopic studies were performed by Dr E. Robertson.

2.4 Screening of the mutant populations

2.4.1 Screening the EMS-mutagenised Arabidopsis *population*

The mutants *arc*1, *arc*2, *arc*3, *arc*1/*arc*5 double mutant and *arc*5 used in this study were originally isolated from an EMS-mutagenised population of Landsberg *erecta Arabidopsis* seeds (Pyke and Leech, 1991)

2.4.2 Screening the T-DNA-mutagenised Arabidopsis *population*

The screen for the T-DNA mutagenised mutants was undertaken with Dr Kevin Pyke. Seeds from the 49 mutagenised pools were sown in wire grids. Forty eight seeds from each of two pools were sown per tray; one seedling per square. The first leaves of individual seedlings were harvested after 23 days growth and transferred to a 96 well ELISA plate for ease of reference between samples and individual plants and prepared as in 2.3.1. The leaf samples were then screened microscopically by eye for putative mutant phenotypes which exhibited significantly different chloroplast numbers or sizes from wild type. Putative mutant plants were saved and allowed to set seed and the mutant phenotype confirmed in this next generation by microscopical analysis of several first leaves from putative mutant seedlings.

2.4.3 Screening the transposon-mutagenised Arabidopsis *population*

Seed was collected by Tanya Page and Anuj Bhatt (Cambridge Lab, John Innes Centre, Norwich, UK) from individual Full Greens (2.1.1) of each *Ac* transformant which represented germinal excision events of the *Ac* (Dean, Sjodin, Page, Jones and Lister, 1992). The Full Greens were heterozygous for the *Ac* element, therefore only 25%

of the seedlings would be homozygous for the *Ac* element and would be likely to display a mutant phenotype. In order to maximise the efficiency of identifying an *arc* phenotype in the progeny of the Full Green, approximately 12 seeds from one Full Green of each line were sown and the seedlings analysed for mutant *arc* phenotypes. Seedlings from 350 lines were screened microscopically (as in 2.3.1) by eye by Dr K. Pyke and one mutant line identified. The seed was saved from each of 12 individual plants in the mutant family, and the progeny seedlings were analysed for their chloroplast phenotype in order to determine the genotype of each of the 12 individual parent plants.

2.4.4 Backcrossing of mutant plants to wild type

Each individual mutant was back-crossed to wild type plants to determine the Mendelian characteristics of the mutant. Crosses were carried out to wild type plants of different ecotype to that of the mutant to facilitate subsequent mapping by RFLP analysis. The backcross to wild type was carried out reciprocally to resolve whether the genetic characteristics of the mutant followed the autosomal inheritance pattern of a nuclear gene rather than maternal inheritance noted in chloroplast genomes in *Arabidopsis* since *Arabidopsis* chloroplasts are maternally inherited. F_1 generation seed from individual siliques of each cross were sown and the *arc* phenotype of each seedling analysed microscopically. 100% wild type seedlings in the F_1 progeny from each reciprocal backcross indicated that the *arc* mutation was a recessive nuclear allele. The bulk F_2 seed obtained from F_1 plants was sown and the mutant chloroplast phenotype of *c.*20 seedlings analysed microscopically for an expected 3:1 ratio of wild type : mutant phenotypes. The result of a 3:1 ratio confirmed the normal Mendelian characteristics of a recessive nuclear mutation.

2.4.5 Allelic crossing between mutant genotypes

Outcrossing experiments between mutants were carried out to test for allelism between mutant phenotypes. A 100% wild type *arc* phenotype in the seedlings of the F_1 generation of the cross indicated that the two mutants were not allelic. Non-allelic mutants were named according to the directions of the *Arabidopsis* multinational steering committee (The Multinational co-ordinated *Arabidopsis thaliana* research project progress

report: year 3; 1994). Non-allelic *arc* mutants were given an *arc* locus number, e.g.. *arc*1; allelic mutants were also numbered with their allele number, e.g.. *arc*6-1 and *arc*6-2

2.5 Image analysis of fixed *Arabidopsis* leaf tissue

2.5.1 Characterisation of mesophyll cell and chloroplast plan area

The detailed analysis of the *arc* mutant cellular phenotypes was facilitated by the use of an image analysis system (Seescan Imaging Ltd, Cambridge, UK) linked to a CCD camera mounted on a Nikon Optiphot microscope (Pyke and Leech, 1987). The images viewed under the microscope could then be transferred to the computer screen and analysed digitally, facilitating the calculation of cell plan area or chloroplast plan area. Primary measurements taken were chloroplast number, mesophyll cell plan area and chloroplast plan area in units of μm^2. Total chloroplast area (see 3.3.2*(iii)*) was derived from the product of the chloroplast number and mean chloroplast plan area for each cell.

2.5.2 Analysis of the frequency of chloroplast division

The analysis of the proportion of chloroplasts in division (4.2.4) was undertaken on mesophyll cells within the leaf cell suspension. Only those chloroplasts whose outline was clearly visible in the plane of focus were included in calculation of the proportion of chloroplasts exhibiting a division profile. A division profile was taken as being an invagination of the chloroplast membrane caused by the central constriction of the membrane rather than the effect of folding of the chloroplast upon itself.

2.6 Preparation of *Arabidopsis* plant genomic DNA

2.6.1 Plant Genomic DNA large-scale preparation

This method of isolating plant genomic DNA was obtained from Dr Ian Bancroft (Cambridge Lab, John Innes Centre, Norwich, UK) modified for *Arabidopsis* from the protocol of Janice Keller (DNAP, Oakland, USA). The protocol produces a yield of approximately 5µg of DNA per gram of leaf tissue. All equipment and aqueous solutions were sterilised, either in a 160°C oven overnight or in an autoclave (10 minutes purge, 20 minutes sterilisation (121°C), 20 minutes cooling). Pestles and mortars were scrubbed in detergent and rinsed thoroughly before sterilisation at 160°C. All macrocentrifugations

were carried out in a Sorvall RC-5B centrifuge with an HS4 rotor, cooled to 4°C. Microcentrifugations were carried out in an Eppendorf 5414 microcentrifuge at 13 500g.

A bulk harvest of leaf material was taken when the plants reached approximately 25 days old, before senescence of the leaves. Approximately 5g of fresh leaf tissue was taken from the plants and crushed in liquid nitrogen with a pestle and mortar. The pestle and mortar were pre-chilled with liquid nitrogen before maceration of the leaves and a fresh pestle and mortar were used for each DNA preparation. The leaves were ground to a fine, dry-looking light green powder, then transferred to 25ml CTAB buffer (140mM sorbitol, 220mM tris pH8, 22mM Na_2EDTA, 800mM NaCl, 1% (w/v) sarcosyl, 0.8% (w/v) CTAB; autoclaved to sterilise) at 65°C in a sterile 50ml disposable centrifuge tube. Optimum DNA extraction was obtained if the powdered leaf material thawed only on contact with the CTAB buffer, therefore a liquid nitrogen-chilled spatula was use to transfer the leaf powder to the CTAB buffer. The buffer was shaken gently to mix the leaf material and placed at 65°C for 20 minutes, shaking gently every 3 minutes. The suspension of leaf material in CTAB was added to 10ml chloroform and shaken on an orbital shaker for 20 minutes at room temperature. The solution was then centrifuged at 1700g for 10 minutes. The clear supernatant containing the DNA in solution was carefully removed and added to a new centrifuge tube containing 17ml ice-cold isopropanol to precipitate the DNA and left for 20 minutes on ice. The sample was centrifuged at 1700g for 10 minutes to pellet the DNA. The supernatant was drained and the walls of the tube dried with tissue paper to remove excess isopropanol . The DNA pellet was resuspended in 4ml sterile Tris EDTA (TE; 10mM Trizma base, 1mM Na_2EDTA, pH to 8.0 with HCl) then mixed with 4ml 4M LiAc and placed on ice for 20 minutes. The sample was spun at 1700g for 20 minutes and the DNA-containing supernatant was drained and added to 16ml ice cold Ethanol and placed on ice for 20 minutes to precipitate the DNA. The ethanol precipitate was spun at 3100g for 20 minutes to pellet the precipitated DNA. The ethanol supernatant was drained and the DNA pellet resuspended in 0.9ml TE. The DNA could now be left overnight at 4°C if necessary.

The DNA solution was treated with phenol to remove remaining proteins from the DNA. 0.1ml 3M NaAc was added to aid the precipitation of the DNA, the sample was

divided equally into two 1.5ml microcentrifuge tubes and mixed with 0.5ml (or an equal volume to the DNA solution) phenol. The covered microcentrifuge tubes were inverted several times until the DNA solution and phenol mixed to an emulsion, then spun in a microcentrifuge at 13 500g for 10 minutes. The DNA-containing phase was the colourless phase, usually the upper of the two phases in the tube. This was removed to a second set of tubes containing 0.5ml 1:1 phenol/chloroform mix, emulsified and microcentrifuged as before. The upper phase was removed and added to 0.5ml chloroform, mixed and spun at 13 500g for 5 minutes, the upper phase was again removed and retained. The yield of DNA was greatly increased by a second extraction using 1 ml of fresh TE processed through the phenol, phenol/chloroform and chloroform solutions discarded from the initial extraction. The DNA-containing fraction of the phenol/chloroform extraction was mixed in microcentrifuge tubes with a double volume of ice-cold 100% ethanol and placed on ice for 20 minutes to precipitate the DNA. The DNA precipitate was pelletted in a microcentrifuge for 20 minutes, the ethanol was drained and the DNA pellet dried (preferably under vacuum) for 10 minutes. The pellet was resuspended in 50-200µl of sterile TE for later analysis and quantitation. The DNA was stored frozen at -20°C.

2.6.2 Plant genomic DNA small-scale preparation from the leaves of a single Arabidopsis *plant*

Extraction of DNA from individual plants required a scaled-down version of the protocol detailed in 2.6.1. As many leaves as possible were taken from a single plant, leaving only enough leaves to allow the plant to set seed. Removal of the first young inflorescence of the plant stimulated extra growth of leaf material, providing a better yield of leaf tissue. Approximately ten leaves yielded 6-8µg DNA. The leaves from each plant were crushed in liquid nitrogen as in 2.6.1, and the powder decanted into 4 sterile 1.5ml microcentrifuge tubes filled with 1ml CTAB buffer at 65°C. After 20 minutes at 65°C, with intermittent shaking, 400µl chloroform was added to each tube and the samples shaken at room temperature for 20 minutes. The samples were then spun in a microcentrifuge at 13 500g for 5 minutes. The supernatants were removed to 4 fresh microcentrifuge tubes each containing 680µl cold isopropanol and left on ice for 20 minutes before spinning in a microcentrifuge at 13 500g for 5 minutes. The pellets were

resuspended in 125µl TE (pH 8) and decanted into two Eppendorf tubes. 250µl 4M LiAc was added to each tube, mixed by gentle inversion and the samples placed on ice for 20 minutes. The samples were spun as before for 5 minutes and the supernatants added to two microcentrifuge tubes containing 1ml ice cold ethanol, left for 20 minutes and spun in the microcentrifuge for 20 minutes to pellet the DNA. The DNA pellets were resuspended in 225µl TE per tube and combined into one of the two tubes. 50µl 3M NaAc was added to the DNA solution and a phenol chloroform extraction carried out as detailed in 2.6.1. Back extraction of the phenol/chloroform series with 500µl TE, used to flush the second microcentrifuge tube used in the ethanol precipitation, greatly enhanced the yield of DNA.

2.6.3 Semi-quantitation of genomic DNA

The visual comparison of the intensity of Ethidium Bromide fluorescence of 2µl of DNA sample against that of known quantities of λ phage DNA was used for the approximate quantitation of DNA to the nearest 50ng. The DNA samples were run uncut on a 1% agarose gel at 40mV for approximately 1 hour. The agarose gel was subsequently stained in 2mg/l ethidium bromide for 10 minutes, followed by destaining in 0.01M $MgSO_4$ for 20 minutes. DNA samples were visualised by illumination of the stained agarose gel on a UV light-box. Ethidium bromide-stained DNA fluoresces pink and may be compared for intensity by eye.

2.7 Restriction enzyme digestion and agarose gel electrophoresis of DNA

2.7.1 Restriction enzyme digests of Plasmid DNA

The digestion of plasmid DNA was usually carried out in a total volume of 30µl, although the reaction may be performed with larger or smaller volumes. The restriction enzyme was stored at -20°C, removed from those conditions for the minimum time before use and, when used, was kept on ice at all times. DNA samples were also kept on ice during preparation of the digestion mixture. The DNA was mixed in a sterile, screw-topped microfuge tube with the appropriate amount of the restriction enzyme buffer supplied with the restriction enzyme (10 fold concentration) and sufficient sterile double distilled water to make up to the final concentration. Finally, 1 unit of restriction enzyme

per μg DNA was added to complete the digestion mixture. The digestion mixture was incubated in a 37°C oven or constant temperature room overnight and a further 1 unit/μg DNA of restriction enzyme was added and the sample incubated in a 37°C heating block for 1 hour. Cut and uncut DNA samples were run on a 1% agarose gel at 40mV for 1 hour, stained with ethidium bromide and visualised under UV light to test for a difference in mobility of the two samples- uncut plasmid DNA migrating further than cut DNA.

2.7.2 Restriction enzyme digestion of Arabidopsis *genomic DNA*

The restriction of *Arabidopsis* genomic DNA was carried out in a volume of 30μl with up to 2 μg DNA, 10 fold concentration restriction enzyme buffer; 1 μg BSA, 1μg Spermidine and 6-10 units of restriction enzyme per μg DNA. The sample was mixed without vortexing and incubated in a 37°C oven or constant temperature room overnight. Spermidine and BSA solutions were prepared in sterile double distilled water and filter sterilised prior to storage at -20°C.

2.7.3 Agarose gel electrophoresis

Agarose gels were prepared as detailed in Sambrook, Fritsch and Maniatis (1993) and performed using an LKB 2013 miniphor and LKB 2012 maxiphor submarine gel tanks. Agarose was mixed with tris borate EDTA (TBE) (prepared as 5 fold concentration stock; diluted to 1 fold concentration with deionised water) and melted in a microwave at medium power until clear. The molten agarose was allowed to cool to approximately 60°C before pouring. DNA samples mixed with 6 fold concentration loading dye (50% (v/v) glycerol, 5mM EDTA, 0.04% (w/v) bromophenol blue, 0.04% (w/v) xylene cyanol, 10mM NaCl) were loaded into the gel and a high voltage applied until the loading dye had migrated from the loading wells of the gel. Minigels were run in at 80V, maxigels at 200V. The voltage was then reduced to 30V for the appropriate period of time for the loading dye to migrate fully.

The majority of agarose gel electrophoresis of DNA was carried out using gel concentration of 1% (w/v) agarose in tris borate EDTA . In some cases a lower concentration of agarose was used. Visualisation of small fragments of DNA, such as

IPCR products was achieved using a 2% agarose gel containing 0.02µg ethidium bromide per ml of agarose solution.

Agarose gels were stained in deionised water containing 2mg/l ethidium bromide for 20 minutes, followed by destaining in 0.01M $MgSO_4$ for 30 minutes. Ethidium bromide-stained DNA was visualised by fluorescence of the ethidium bromide on a UV transilluminator.

2.8 Southern blotting and slot blotting of genomic DNA

2.8.1 Origin of DNA probes used in southern blot hybridisations

The Left Border, Right Border and pBR322 probes used in section 3.1.3 were a kind gift of Dr M. Bennett, University of Warwick, UK. The *Ac* and *SPT* gene probes were kindly donated by Dr Anuj Bhatt, Cambridge Lab, John Innes Centre, Norwich, UK. The ARMS probes were donated by Dr A. Schäffner *via* the *Arabidopsis* Biological Resource Centre, Columbus, Ohio, USA (Stock Number: CD-46 to CD-76).

2.8.2 Southern blotting of restriction enzyme-digested Arabidopsis *genomic DNA*

The southern blotting protocol was modified by Dr Clare Lister from the Amersham International Hybond-N instruction leaflet (Amersham International Ltd, Amersham, UK).

1-3µg genomic DNA per sample was cut as detailed in 2.7.2 and run on a submarine 0.7% agarose gel overnight at 25-40V. One track in the gel was loaded with 0.2µg Lamda DNA cut with *Eco*RI and *Hin*dIII as a size marker lane. The gel was stained in 2mg/l ethidium bromide and photographed on a UV transilluminator. After photography of the gel, the bottom left hand corner of the gel was cut away for the subsequent orientation of the gel during the blotting process. The areas of the gel not containing DNA were removed before blotting.

The gel was treated to depurinate and denature the DNA before blotting. The gel was depurinated in 500ml 0.25M HCl, shaking gently for 2 x 15 minutes, and then flushed with 500ml deionised water and drained immediately. The gel was then denatured in 500ml 1.5M NaCl, 0.5M NaOH for 2 x 15 minutes and flushed with deionised water.

The gel was finally neutralised by treatment with 2x500ml neutralisation solution (1.5M NaCl, 1.0M Tris, pH8 with HCl) for 15 minutes each.

The DNA was transferred by capillary blotting to Hybond-N (0.45 μm) nylon membrane (Amersham International Ltd, Amersham, UK). The membrane was labelled in black ball-point pen before blotting and the bottom left hand corner of the filter was cut off to mimic that of the gel. A glass plate was placed over a reservoir of 20X SSC (3M NaCl, 0.3M Na_3Citrate). A wick of four sheets of No1 Whatman filter paper were soaked in SSC and draped over the glass plate with at least 100mm of the wick immersed in the reservoir on either side of the glass plate. Each successive layer of the capillary blot apparatus was placed individually and air bubbles smoothed out with a sterile glass 10ml pipette. The gel was placed centrally on the wick and exposed areas of the wick were covered with plastic film to avoid contact of the wick with the blotting paper towels. The Hybond-N membrane was cut to the size of the gel, soaked in 20X SSC and placed over the gel. Six sheets of filter paper, cut to the size of the gel and soaked in 20X SSC were placed on the Hybond-N membrane and covered with a stack of paper towels weighted with a 0.5kg weight. The capillary blot apparatus was covered with cling film and left overnight. The capillary blot was dismantled and the membrane peeled away from the gel. The membrane was washed by very gentle rocking in 2X SSC for 30 seconds. The DNA was UV cross-linked to the Hybond-N filter by treating DNA side up with 150mJ ultraviolet energy in a Biorad GS Genelinker (Biorad, Hemel Hempstead, UK), at the 'C3' setting. The filter was finally dried, then placed between two sheets of filter paper and baked at 80°C for 5 hours. The filter could then be stored dry at room temperature.

2.8.3 Slot blotting of denatured Arabidopsis *genomic DNA*

Slot blotting was performed with the Biodot SF blotting apparatus (Biorad, Richmond, UK), slot blot format. Genomic DNA diluted in 200μl sterile double-distilled water was denatured by boiling for 5 minutes followed by ice for 10 minutes. The DNA solution was applied to the wells of the slotblot apparatus containing Hybond N nylon membrane (2.8.2) moistened with 20X SSC. All unused wells were filled with 200μl sterile water and a gentle vacuum applied to the apparatus to aid the draining of the wells.

The wells were flushed with 200µl 2X SSC and drained as before. The apparatus was dismantled and the Hybond-N membrane treated as detailed in 2.8.2.

2.8.4 Preparation of ^{32}P dCTP-labelled DNA probe

DNA probes for Southern blot hybridisation were prepared using 50-100ng of probe DNA labelled by incorporation of ^{32}P-labelled dCTP, using an Oligolabelling kit (Pharmacia Biotech, St Albans, UK). Less than 100ng DNA diluted in sterile double distilled water to a total volume of 34µl was denatured by boiling in a waterbath for 3 minutes, followed by 3 minutes on ice. 10µl reagent mix (dATP, dGTP, dTTP and random hexadeoxyribonucleotides in buffer), 1µl (5-10units) DNA polymerase Klenow fragment and 5µl (50µCi) 3000 Ci/mmol $a^{32}P$ dCTP were added to the denatured DNA and incubated at 37°C for 3 hours. The unincorporated DNA was removed by elution through a Sephadex G50 gel column (Nick column, Pharmacia Biotech). The labelled probe solution was denatured by boiling for 5 minutes, followed by ice for 15 minutes before hybridisation to the Southern blot filter.

2.8.5 Hybridisation of the ^{32}P-labelled DNA probe to the southern blot filter

Hybridisation of the probe to the DNA-bound nylon Southern blot filter was carried out as suggested in the Hybond-N instruction leaflet, modified by C. Lister. The filter was incubated with 20ml hybridisation buffer (0.5% (w/v) SDS, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, 0.1% (w/v) PVP in 5X SSC), warmed to 65°C, and 1mg herring sperm DNA (5mg/ml stock, dissolved at 37°C shaking overnight, pipetted for 30 minutes through a number 18 syringe needle) denatured by boiling for 5 minutes; in a sealed glass tube. The tube was rocked gently to moisten the filter before incubation, rotated at 65°C in a rotating hybridisation oven for 3 hours. The hybridisation mixture was then replaced by 3-4ml fresh hybridisation buffer, warmed to 65°C with 50µg/ml hybridisation buffer of denatured herring sperm DNA. The denatured probe was added to the hybridisation tube and the sealed tube incubated overnight, rotating at 65°C.

The hybridised filter was washed to remove excess radioactivity and non-specifically bound probe prior to autoradiography. The hybridisation buffer (2.8.5),

including radioactive probe, was drained and replaced by 100ml 2X SSC. The hybridisation tube was gently rocked for 30 seconds before the 2X SSC was drained and replaced with 100ml low stringency wash solution (2X SSC, 1% (w/v) SDS, warmed only enough to allow the SDS to dissolve) and incubated with rotation at 65°C for 30 minutes. The low stringency wash was replaced by a fresh 100ml of low stringency wash and incubated for a further 30 minutes. The filter was then removed from the glass tube and placed flat on a plastic sheet and checked with a high-sensitivity Geiger counter. If there was sufficiently low amounts of background radioactivity on the filter, it was sealed in a plastic bag for autoradiography, otherwise, it was washed with high stringency wash solution. The high stringency wash (0.1X SSC, 1% (w/v) SDS) was carried out at 65°C for up to 20-30 minutes, repeating if necessary until background activity was reduced. The washed filter was sealed in a plastic bag to prevent drying of the filter. The filter was required to be kept moist if it was to be stripped and reprobed after autoradiography.

The DNA probe could be stripped from a moist filter by shaking in 0.4M NaOH at 45°C for 30 minutes, followed by stripping solution (0.1X SSC, 0.1% (w/v) SDS, 0.2M Tris HCl at pH7.5) shaking at 45°C for 15 minutes. The filter could then be stored dry before reprobing.

2.8.6 Autoradiography of the ^{32}P-labelled Southern blot filter

The labelled Southern blot filter sealed in a plastic bag was exposed at -80°C to Kodak XAR5 (X-omat) X-ray film (Sigma, UK) in a Hypercassette autoradiography cassette fitted with two Hyperscreen intensifying screens (Amersham International, Amersham, UK). The film was developed, under safelight conditions, by shaking in Kodak D-19 developer for 6 minutes, followed by 20% Kodak Unifix X-ray film fixative for twice the clearing time of the autoradiograph.

2.9 Amplification, cloning and sequencing of plasmid and *Arabidopsis* plant genomic DNA

2.9.1 Amplification of plant DNA sequences by Polymerase Chain Reaction (PCR)

The PCR protocol used follows that of Jarvis, Lister and Dean (1994). The PCR procedure requires stringent precautions against contamination by foreign DNA or plasmid DNA. Fresh, autoclaved microfuge tubes and pipette tips were used at all times; all solutions used were dedicated solutions for PCR only; the working area and Gilson pipettes were cleaned thoroughly with 1% Decon, followed by water and finally ethanol; gloves were changed frequently.

Genomic DNA was digested with restriction enzyme overnight at 37°C. 50ng of digested DNA was used for the PCR reaction. The DNA was added to 25nM dNTPs (equal concentrations), 25nM of each PCR primer, 10X buffer, 2.5 units Taq polymerase and sterile double distilled water in a total volume of 100µl. Reactions were mixed in sterile 500µl microfuge tubes and covered with 150µl mineral oil to prevent evaporation during the PCR process. The PCR reaction was incubated in a Peltier Thermal cycling machine. The reactions were heated at 95°C for 5 minutes, followed by 50 cycles of 95°C for 30 seconds, 56°C for 30 seconds and 72°C for 3 minutes. Reactions were cooled to 4°C after the completion of the programme.

2.9.2 Amplification of plant DNA sequences flanking the Ac element by Inverse Polymerase Chain Reaction (IPCR)

Genomic DNA from the *arc*11 mutant, 5 mutant F_2 siblings and wild type control was digested with *Bst*YI and *Bcl*I restriction enzymes, which both cut within the *Ac* element and would also cut externally at an unknown site within the plant flanking DNA. Restriction enzyme digests were performed individually and the first enzyme was removed from the DNA by extraction with phenol/chloroform mix and ethanol precipitation before the second enzyme was added. A double digest was performed to attempt to reduce the size of the restriction fragments for the IPCR reaction for increased efficiency of the IPCR

reaction. 2.5µg of genomic DNA was cut in a total volume of 25µl reaction mixture and digested to completion overnight at 37°C.

IPCR was performed on circularised DNA restriction fragments. The restriction enzyme-digested DNA was circularised by ligation in a large reaction volume to stimulate intraspecific ligation of the DNA fragments. 10 µl (1µg DNA) of the digested DNA was added to 40µl 10X Ligase buffer, 1.5µl (10 units) DNA Ligase, made up to 400µl with fresh, sterile double distilled water. The ligation reaction was incubated overnight at 16°C.

0.02µg Yeast tRNA and 40µl sterile 3M sodium acetate was added to the ligation mixture to aid precipitation of the DNA. The ligase was removed from the DNA solution by mixing with 450µl phenol-chloroform, after spinning in a microfuge for 5 minutes the upper phase was removed to 1ml cold ethanol and the DNA was precipitated at -70°C for 30 minutes. The precipitated DNA was spun for 10 minutes in a microcentrifuge and the ethanol drained. In order to reduce the salt concentration in the DNA pellet, 500µl 70% ethanol was added gently and the tube respun for 2 minutes. The 70% ethanol was drained and the DNA pellet dried under vacuum then redissolved in 10µl sterile double distilled water. A minigel of 2µl of ligated DNA after precipitation, was run to check that the ligation had worked. The unligated control DNA should migrate further in the gel than ligated DNA.

50ng of circularised DNA was used for the IPCR reaction, following an identical protocol to 2.9.1.

2.9.3 Preparation of competent E. coli *cells for transformation of DNA*

JM101 *E. coli* cells were grown in 5ml nutrient broth, shaking at 37°C from a single colony to $OD_{550} = 0.3$. The bacterial suspension was transferred to 100ml pre-warmed nutrient broth, shaking at 37°C for 2-3 hours until $OD_{550} = 0.48$. The cells were chilled on ice and spun in 2x50ml centrifuge tubes at 1200g at 0°C for 10 minutes. The bacterial pellet was gently resuspended in 30ml ice cold transformation buffer I (30mM KOAc, 50mM $MnCl_2$, 100mM RbCl, 10mM $CaCl_2$, 15% (w/v) glycerol; pH 5.8 with 0.2M HOAc; filter sterilised and stored at 4°C) and incubated for 120 minutes on ice. The bacterial suspension was spun at 1200g and resuspended gently on ice in 4ml transformation buffer II (10mM NaMOPS (pH7.0), 75mM $CaCl_2$, 10mM RbCl, 15%

(w/v) glycerol), filter sterilised and stored at 4°C). Cells were aliquotted into ice cold microfuge tubes and stored at -70°C.

2.9.4 Transformation of competent JM101 E. coli *bacterial cells with plasmid DNA*

An aliquot of the competent cells (2.9.3) was thawed on ice. A volume of 25µl containing not more than 100ng DNA was added to 200µl cell suspension. The DNA and cell suspension were mixed by inversion of the microfuge tube and incubated for 20 minutes on ice. The cells were heat shocked for 120 seconds at 37°C before spreading on selective medium nutrient agar plates and incubated for 16 hours at 37°C.

2.9.5 Isolation of plasmid DNA from transformed host bacterial cells

The isolation of plasmid DNA from transformed bacterial cells follows the protocol of C. Lister (unpublished).

Transformed bacteria from a single colony were cultured in nutrient medium containing selective antibiotic overnight, shaking at 37°C. 1.5ml culture was removed to a microfuge tube and spun at 13 400g for 5 minutes. The pellet was resuspended in 350µl boiling buffer (8% (w/v) sucrose, 0.5% (v/v) triton X-100, 50mM NaEDTA (pH8), 10mM Tris HCl (pH8); autoclaved to sterilise), 0.25µl of 10mg/ml lysosyme (prepared fresh in TE and kept on ice) was added, the suspension mixed by inversion and incubated at room temperature for 5 minutes. The mixture was boiled for 1 minute, cooled on ice, then spun at 13 500g for 20 minutes. The mucilaginous pellet was removed with a sterile toothpick, 40µl 3M NaAc and 220µl isopropanol added to the mixture. The tube was inverted several times before spinning at 13 000 rpm for 5 minutes to pellet the DNA. The pellet was dried under vacuum before resuspension in 40µl TE.

2.9.6 Isolation of cloned DNA from plasmid vector DNA

The isolation of cloned fragments from agarose gels was carried out by removal of the relevant band from an agarose gel electrophoresis of digested plasmid DNA. The agarose containing the DNA was removed to a sterile 1ml disposable syringe and frozen overnight at -20°C. The frozen agarose was gently warmed in the hands whilst being compressed by the syringe. The liquid contents of the agarose, containing the DNA in

solution, was collected; a 10% volume of 7.5M NH_4Ac was added to the DNA solution and the DNA precipitated by the addition of twice the volume of ethanol at -20°C overnight.

2.9.7 Sequencing of DNA fragments obtained from IPCR of flanking plant DNA sequences to the Ac *element*

Sequencing of DNA fragments was carried out by Dr C. Lister using the ^{T7}Sequencing kit (Pharmacia Biotech, St Albans, UK). The protocol for the sequencing reaction was unaltered from the ^{T7}Sequencing kit instruction leaflet using ^{35}S radioactive label. The sequencing was carried out on a polyacrylamide gradient sequencing gel (Sambrook, Fritsch and Maniatis, 1993).

CHAPTER 3

The Isolation of *arc* Mutants in *Arabidopsis thaliana*

3.1 INTRODUCTION

3.1.1 The use of insertional mutagenesis to facilitate the isolation of ARC *genes by gene tagging*

The EMS mutagenised *arc* mutants isolated by Pyke and Leech (1992, 1994) are of significant value to the analysis of chloroplast division in *Arabidopsis*; they are not, however, ideal for the isolation of the *ARC* genes which they represent. This is because the EMS mutagen commonly induces mutation by single base alterations (Redei, 1992), requiring a map-based cloning strategy of 'chromosome walking', using complex RFLP analysis to isolate the mutagenised gene locus (Gibson and Somerville, 1992). This approach was not favoured for the isolation of *ARC* genes due to the extremely labour-intensive nature of the technique and because there is no sufficiently accurate genetic map of the *Arabidopsis* genome presently available. An alternative approach to chromosome walking involves the use of 'gene tagging', a technique in which a mutant phenotype is induced by the insertion of a DNA fragment of a known sequence which may then be used to identify the mutagenised locus. Once an appropriate phenotype has been shown to be tagged DNA probes which recognise the inserted sequence can be used to isolate and amplify the plant genomic DNA flanking the inserted DNA by a variety of methods which are discussed below. The use of gene tagging has already facilitated the isolation of several genes in *Arabidopsis*, such as the *GLABROUS* 1 gene (Marks and Feldmann, 1989), the *DWF* locus (Feldmann, Marks, Christianson and Quatrano, 1989), the *DRL*1 locus (Bancroft, Jones and Dean, 1993), and various *meri* genes affecting the apical meristem (Medford, Elmer and Klee, 1991); a good review describing the potential of gene tagging for the isolation of genetic loci in *Arabidopsis* is Feldmann, Malmberg and Dean (1994).

The methods of insertional mutagenesis commonly adopted for gene tagging in *Arabidopsis* at present involve the use of one of two systems. *(a)* The T-DNA sequence of the *Ti* plasmid of *Agrobacterium tumefaciens* (André, Colau, Schell, Van Montagu and Hernalsteens, 1986; Koncz, Martini, Mayerhofer *et al* 1989; Feldmann, 1991; Topping, Wei and Lindsay, 1991; Walden, Hayashi and Schell, 1991; Forsthoefel, Wu, Schulz,

Bennett and Feldmann, 1992); *(b)* Transposable elements, such as the *Ac/Ds* system (Dean, Sjodin, Lawson, *et al*, 1990; Coupland, 1992) or the *En/Spm* system of maize (Aarts, Dirske, Stiekema and Pereira, 1993).

3.1.2 Insertional mutagenesis of Arabidopsis *using a modified T-DNA of* Agrobacterium tumefaciens

The T-DNA from the *Ti* plasmid of *Agrobacterium tumefaciens* is the most common means of transformation in dicotyledonous plants at present (Koncz, Schell and Redei, 1992). The insertional mutagenesis of *Arabidopsis* using the T-DNA has been successful in many instances (André, Colau, Schell, Van Montagu and Hernalsteens, 1986; Koncz, Martini, Mayerhofer, *et al,* 1989, Topping, Wei and Lindsay, 1991). However there is one serious disadvantage in this technique, namely that the transformation of *Arabidopsis* with *Agrobacterium* for T-DNA transfer generally requires the use of tissue culture which may lead to serious morphological anomalies in the transformed plant as a result of somaclonal variation (Feldmann and Marks, 1986). The seed transformation protocol developed by Feldmann (Feldmann and Marks, 1987) was designed to alleviate this problem of somaclonal variation by using a seed infection transformation method rather than one involving tissue culture, potentially providing a more stable phenotypic base for the mutagenised populations. The probability of isolating a mutation which was caused by the insertion of a T-DNA rather than by somaclonal variation was predicted to be higher than if tissue culture had been used (Feldmann and Marks, 1987). This increased probability of obtaining a tagged alele would make T-DNA mutagenised plants transformed by seed infection more reliable for the isolation of genes by gene tagging.

The 3850:1003 T-DNA construct used by Feldmann was adapted from the pGV3850 and pAK1003 plasmid constructs of Velten and Schell (1985). The content of the 3850:1003 T-DNA construct is illustrated in figure 3.1, adapted from Errampalli, Patton, Castle *et al* (1991) and Castle, Errampalli, Atherton *et al* (1993). The T-DNA is 17 kb in size and contains left and right border sequences to facilitate the integration of the T-DNA into the host genome. Two sequences from the autonomously replicating plasmid

pBR322 (3.8 kb and 4.4 kb respectively) are also included in the T-DNA to enable the replication of fragments of T-DNA and plant flanking DNA during the amplification of a tagged locus *via* plasmid rescue (Herman and Marks, 1989). Nopaline synthetase (*NOS*), and Neomycin phosphotransferase (*NPTII*, conferring resistance to the antibiotic kanamycin) genes are also included with their respective promoters (Velten and Schell, 1985; Forsthoefel, Wu, Schulz, Bennett and Feldmann, 1992) to select for the presence of the T-DNA in plants (NPTII driven by the 1' promoter of the pTiAch5 plasmid; and NOS) and bacteria (NPTII). Figure 3.1 also indicates restriction enzyme sites for *Hin*dIII, *Eco*RI and *Sal*I.

The seed infection protocol, adapted from Forsthoefel, Wu, Schulz, Bennett and Feldmann (1992) is illustrated in figure 3.2 for the production of a single mutagenised line of seeds. Wild type seed was co-cultivated with *Agrobacterium tumefaciens* and transformed with the 3850:1003 T-DNA construct. The (T_1 generation) seed was germinated and grown to maturity and bulk T_2 generation seed obtained collectively from all plants. Incorporation of the T-DNA into the host genome is suggested to occur during gametogenesis of the T_1 plant (Feldmann, 1991). The T_2 seedlings were tested for the presence of a T-DNA in the genome by resistance of the seedling to kanamycin conveyed by the 1' NPTII gene of the T-DNA construct. Kanamycin resistant seedlings were heterozygous for the T-DNA, due to the integration of the T-DNA into only one chromosome of the T_1 plant. T_3 seed was saved in bulk from the kanamycin resistant (kanR) T_2 plants. This seed segregated for kanR/kanR, kanR/kanS and kanS/kanS phenotype. Each line contains an assortment of individual tranformant plants, segregating in the T_3 generation for the mutation and kanamycin resistance.

Sub pools of seed were generated by the collective cultivation of 20 T_3 seeds per transformed line from a total of 20 lines. The bulk harvest of seeds from these 400 plants was collected as a *sub pool* which represented 20 separate transformed lines. Equivalent numbers of seeds from each of 5 lines were collected as a *pool* of seed from 100 lines. The screen for T-DNA mutagenised *arc* mutants used 49 pools of 100 lines each, representing 4900 individual transformed lines, each segregating for several separate T-DNA insertion events.

Figure 3.1

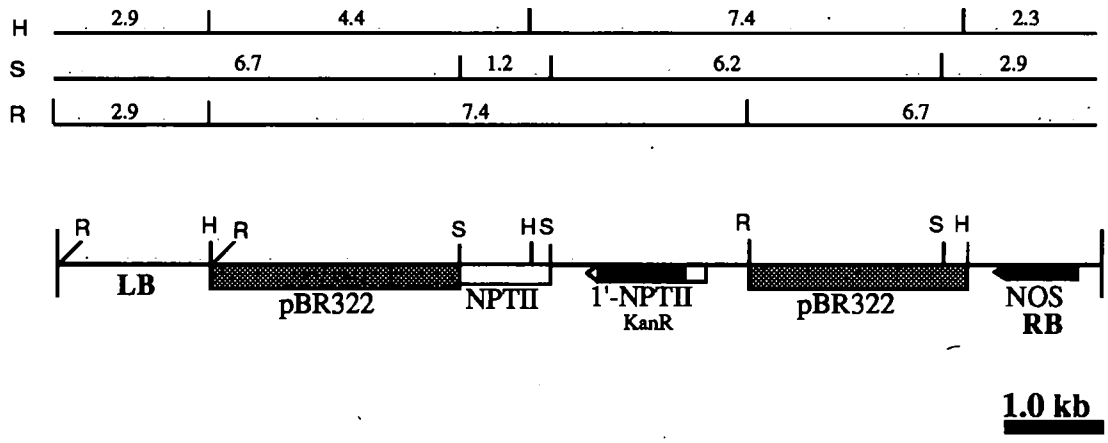


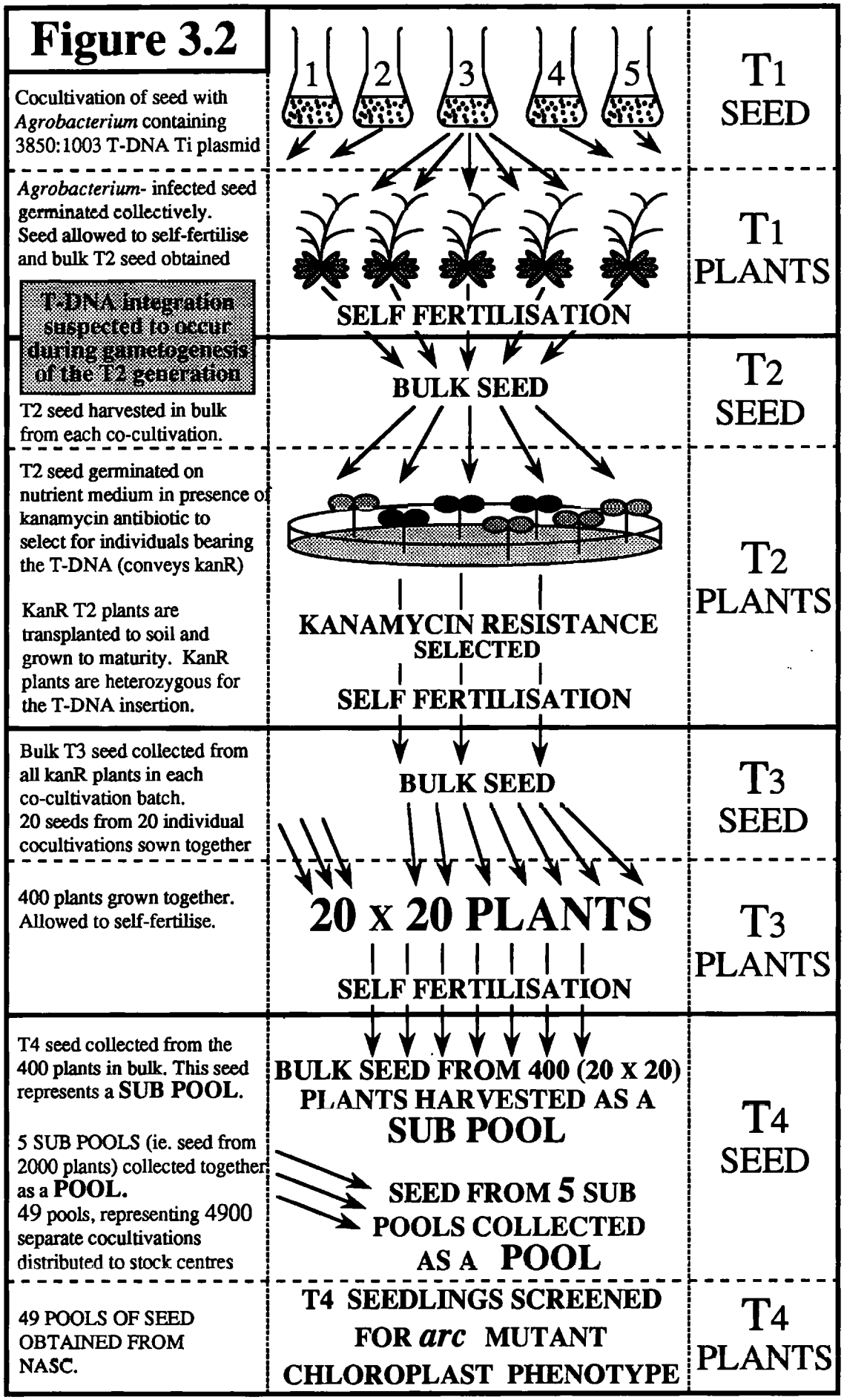
FIGURE 3.1 The 3850:1003 T-DNA construct

Diagrammatic representation of the 3850:1003 T-DNA adapted from (Velten and Schell, 1985; Errampalli, Patton, Castle *et al* (1991) and Castle, Errampalli, Atherton *et al* (1993)). Restriction enzyme sites are indicated for *Eco*RI (**R**), *Hin*dIII (**H**) and *Sal*I (**S**) with restriction fragment sizes indicated in kilobase pairs. **LB** = Left border; **pBR322** = pBR322 autonomous plasmid sequences including origin of replication; **NPTII** = Neomycin phosphotransferase gene for kanamycin resistance in bacteria; **1' NPTII** = Neomycin phosphotransferase gene for kanamycin resistance in plants. **NOS** = Nopaline synthetase gene; **RB** = Right border.

FIGURE 3.2 **The construction of the T-DNA-mutagenised lines**

Diagrammatic representation of the seed infection protocol of Dr K.A. Feldmann (Feldmann and Marks, 1987), used to construct the T-DNA mutagenised lines of *Arabidopsis thaliana* (ecotype WS).

Figure 3.2


Cocultivation of seed with *Agrobacterium* containing 3850:1003 T-DNA Ti plasmid
1
2
3
4
5
T1 SEED
Agrobacterium- infected seed germinated collectively. Seed allowed to self-fertilise and bulk T2 seed obtained
SELF FERTILISATION
T1 PLANTS
T-DNA integration suspected to occur during gametogenesis of the T2 generation
BULK SEED
T2 seed harvested in bulk from each co-cultivation.
T2 SEED
T2 seed germinated on nutrient medium in presence of kanamycin antibiotic to select for individuals bearing the T-DNA (conveys kanR)
KanR T2 plants are transplanted to soil and grown to maturity. KanR plants are heterozygous for the T-DNA insertion.
KANAMYCIN RESISTANCE SELECTED
SELF FERTILISATION
T2 PLANTS
Bulk T3 seed collected from all kanR plants in each co-cultivation batch. 20 seeds from 20 individual cocultivations sown together
BULK SEED
T3 SEED
400 plants grown together. Allowed to self-fertilise.
20 x 20 PLANTS
SELF FERTILISATION
T3 PLANTS
T4 seed collected from the 400 plants in bulk. This seed represents a SUB POOL.
5 SUB POOLS (ie. seed from 2000 plants) collected together as a POOL.
49 pools, representing 4900 separate cocultivations distributed to stock centres
BULK SEED FROM 400 (20 x 20) PLANTS HARVESTED AS A SUB POOL
SEED FROM 5 SUB POOLS COLLECTED AS A POOL
T4 SEED
49 POOLS OF SEED OBTAINED FROM NASC.
T4 SEEDLINGS SCREENED FOR *arc* MUTANT CHLOROPLAST PHENOTYPE
T4 PLANTS

The two pBR322 sequences of the 3850:1003 T-DNA (figure 3.1) each contain an *E. coli* Origin of Replication and an ampicillin resistance marker, allowing for the isolation of plant DNA sequences flanking the T-DNA by plasmid rescue (Herman and Marks, 1989; Koncz, Mayerhofer, Koncz-Kalman *et al*, 1990; Castle, Errampalli, Atherton *et al*; 1993). In the technique of plasmid rescue, restriction enzyme-digested fragments containing T-DNA and flanking plant DNA sequences may be circularised and imported into bacterial host cells. The *E. coli* Origin of Replication of the pBR322 sequence enables the replication of the circularised fragment of T-DNA and genomic DNA within the host cell and is selectable by ampicillin resistance of the transformed host bacterial cell. The amplified plant genomic DNA may then be cloned and sequenced. The use of plasmid rescue requires that the T-DNA which tags the gene of interest be fully intact and functional. A truncated or rearranged T-DNA will probably not contain the requisite sequences for the replication of the transformed bacteria.

3.1.3 Insertional mutagenesis of Arabidopsis *using the* Ac *element of* Zea mays

The *Ac*-mutagenised populations of *Arabidopsis* had been developed in Dr C. Dean's laboratory by the following procedure. The Δ*Nae*I *Ac* transposon was modified from the wild type maize *Ac* element by the deletion with *Nae*I restriction enzyme of a 536bp fragment from the 5' untranslated leader of the transposase mRNA. The *Nae*I deletion caused an increased activity of the *Ac* transposon in *Arabidopsis* (Lawson, Scofield, Sjodin, Jones and Dean, 1994). The modified *Ac* element was cloned into the *SPT* gene of a modified *Agrobacterium* T-DNA, which also contained the *1'NPTII* fusion (Velten and Schell, 1985) for selection of transformed plant material (Bancroft, Bhatt, Sjodin *et al*, 1992). The Δ*Nae*I *Ac* construct was cloned in both orientations within modified T-DNA binary vectors named 02213 and 0383 (Lawson, Scofield, Sjodin, Jones and Dean, 1994). The T-DNA constructs were transformed into plant material, ecotype Landsberg *erecta*, as detailed in Dean, Sjodin, Page, Jones and Lister (1992). Excision of the *Ac* element was characterised by the reactivation of the streptomycin

resistance (*SPT*) gene resident in the T-DNA fusion in which the *Ac* was housed which was identified as a partially green or fully green phenotype in a streptomycin assay (Dean, Sjodin, Page, Jones and Lister, 1992). The progeny of each Full Green, which represented a unique excision event, were screened for *arc* mutant mesophyll cell phenotypes.

Figure 3.3 illustrates the construction of the Δ*Nae*I *Ac* -mutagenised populations. Figure 6.2 in chapter 6 illustrates the composition of the Δ*Nae*I *Ac* element and the SPT:T-DNA used in the mutagenesis of the populations.

The plant DNA flanking a tagged Δ*Nae*I *Ac* -mutagenised locus may be isolated by the technique of 'inverse polymerase chain reaction' (IPCR). The digestion of mutant genomic DNA containing a transposed *Ac* element with *Bst*YI and *Bcl*I provides restriction fragments, containing either left or right border fragments of the *Ac*. By circularisation of these fragments the *Ac* and plant genomic sequences may be amplified by IPCR initialised with probes to the known sequence of the *Ac* borders. The IPCR fragments may then be cloned for the analysis of sequence data of the plant DNA flanking the *Ac*.

3.1.4 Experimental approach to the isolation of tagged arc *mutants*

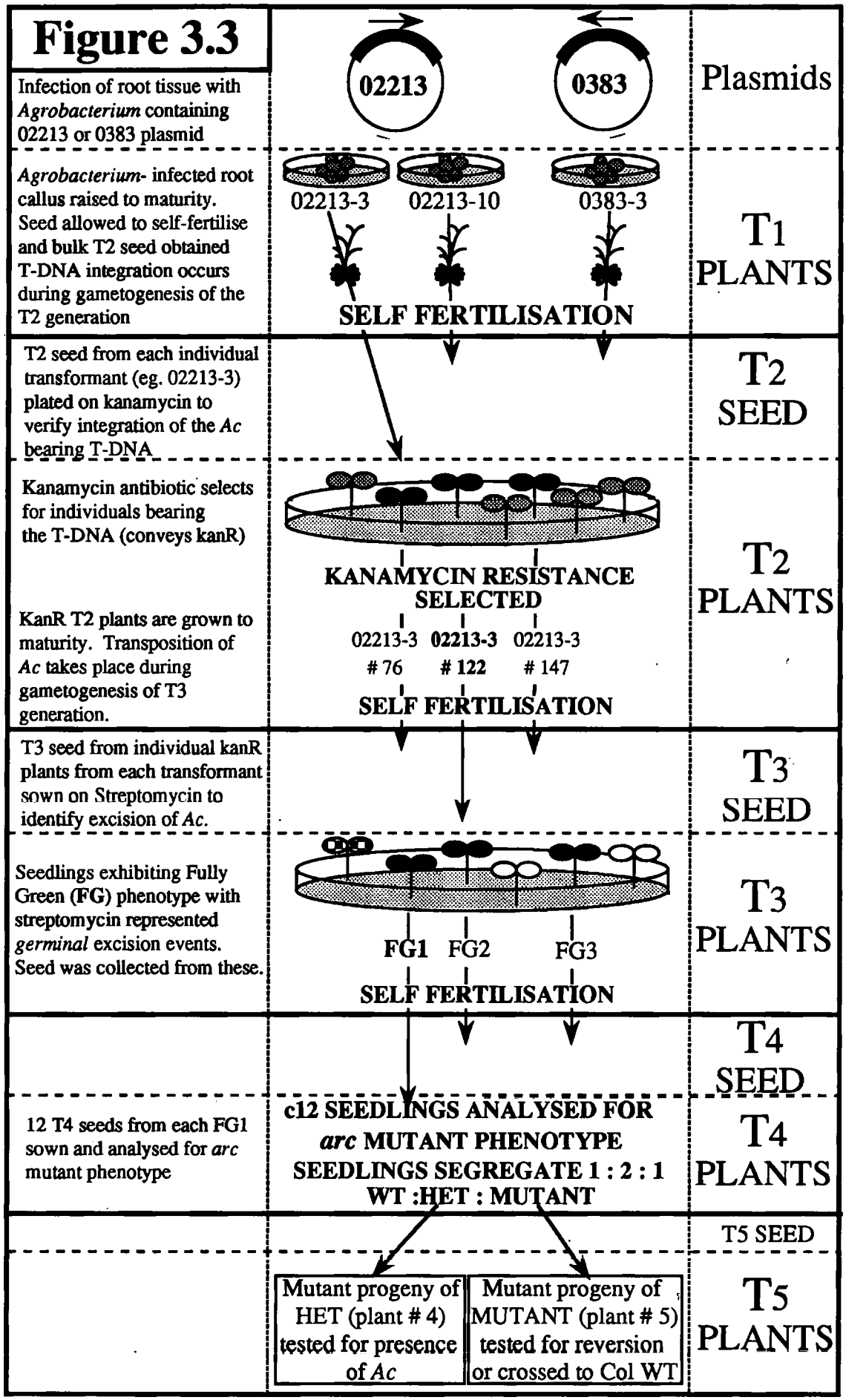
Two populations of *Arabidopsis* mutagenised by DNA insertion were selected to screen for *arc* mutant phenotypes. The first population was the T-DNA-mutagenised families developed by Dr K. Feldmann, University of Arizona, USA, were chosen initially for the mutant screen: These mutagenised lines were donated by Dr Feldmann *via* the Nottingham *Arabidopsis* Seed Centre. The second population used was the Δ*Nae*I *Ac* -mutagenised lines developed by Dr C. Dean and co-workers and were donated directly by Dr C. Dean, Cambridge Laboratory, John Innes Centre, Norwich, UK.

My screen was performed microscopically by eye rather than by using the automated screen developed by Pyke and Leech (1991). I screened for a mesophyll cell chloroplast phenotype which appeared to differ from the wild type phenotype in both chloroplast number and chloroplast size.

FIGURE 3.3 The construction of the Δ*Nae*I *Ac* mutagenised lines

Diagrammatic representation of the transformation protocol of C. Dean and co-workers (Lawson, Scofield, Sjodin, Jones and Dean, 1994) used to construct the 02213 and 0383 Δ*Nae*I *Ac* mutagenised lines of *Arabidopsis thaliana* (ecotype Landsberg *erecta*).

Figure 3.3


Infection of root tissue with *Agrobacterium* containing 02213 or 0383 plasmid
02213
0383
Plasmids
Agrobacterium- infected root callus raised to maturity. Seed allowed to self-fertilise and bulk T2 seed obtained T-DNA integration occurs during gametogenesis of the T2 generation
02213-3
02213-10
0383-3
T1 PLANTS
SELF FERTILISATION
T2 seed from each individual transformant (eg. 02213-3) plated on kanamycin to verify integration of the *Ac* bearing T-DNA
T2 SEED
Kanamycin antibiotic selects for individuals bearing the T-DNA (conveys kanR)
KANAMYCIN RESISTANCE SELECTED
KanR T2 plants are grown to maturity. Transposition of *Ac* takes place during gametogenesis of T3 generation.
02213-3 # 76
02213-3 # 122
02213-3 # 147
SELF FERTILISATION
T2 PLANTS
T3 seed from individual kanR plants from each transformant sown on Streptomycin to identify excision of *Ac*.
T3 SEED
Seedlings exhibiting Fully Green (FG) phenotype with streptomycin represented *germinal* excision events. Seed was collected from these.
FG1
FG2
FG3
SELF FERTILISATION
T3 PLANTS
T4 SEED
12 T4 seeds from each FG1 sown and analysed for *arc* mutant phenotype
c12 SEEDLINGS ANALYSED FOR *arc* MUTANT PHENOTYPE
SEEDLINGS SEGREGATE 1 : 2 : 1 WT :HET : MUTANT
T4 PLANTS
T5 SEED
Mutant progeny of HET (plant # 4) tested for presence of *Ac*
Mutant progeny of MUTANT (plant # 5) tested for reversion or crossed to Col WT
T5 PLANTS

In this chapter the utilisation of these two types of tagged populations for the isolation of mutant chloroplast division phenotypes is described. From the T-DNA mutagenised population, 13 000 individuals were screened revealing 6 mutants representing 5 novel *arc* mutant phenotypes. From the Δ*Nae*I *Ac* -mutagenised population, 350 individual families were screened revealing one novel mutant phenotype. The *arc*11 mutant, isolated from the transposon mutagenised population is the only novel *arc* phenotype isolated which is tagged with a DNA insertion useful for the isolation of the mutant locus. The novel *arc* mutant phenotypes show several similarities to existing *arc* mutants. In all the mutants the increase in chloroplast number and total chloroplast area per cell is closely correlated with increasing mesophyll cell size. A compensation of chloroplast size for perturbed chloroplast number is also observed. The characterisation of the phenotypes of the novel *arc* mutants has already provided considerable insight into the control of the process of chloroplast division in *Arabidopsis*, as described in this thesis.

3.2 RESULTS

3.2.1 A comparison of the whole plant and mesophyll cell phenotypes of the Landsberg erecta *and WS ecotypes of* Arabidopsis thaliana

The EMS and transposon-mutagenised populations were developed in the Landsberg *erecta* (Ler) ecotype of *Arabidopsis*, whereas the T-DNA mutagenised populations were developed in the ecotype Wassilewskija (WS). The Ler and WS ecotypes show several subtle differences in whole plant and mesophyll cell morphology. The wild type mesophyll cell and whole plant phenotypes of the two ecotypes were examined to allow effective comparison of the *arc* mutants which were isolated from the mutagenised populations of both ecotypes.

The Landsberg *erecta* and WS whole plant phenotypes differ in several aspects. Figure 3.4 (*a*) and (*b*) illustrates the whole plant phenotype of typical mature Ler and WS wild type plants. The Ler ecotype exhibits the *erecta* mutation which causes a compaction of the plant phenotype. The Ler seedling is slightly smaller than the WS seedling, and the Ler mature plant is stockier than WS with a floral bolt which is shorter. The floral rosette is also more compact in the Ler ecotype than in WS (Figure 3.4 (*c*) and (*d*)), and the WS plant appears to flower slightly earlier than Ler by approximately 5 to 7 days. The first leaves of the two ecotypes are not significantly dissimilar, however the later leaves of WS ecotype are slightly larger than Ler

A much more significant difference between Ler and WS for the comparison of *arc* mutant phenotypes is evident in the phenotype of the *mesophyll cells* (Figure 3.4 (*e*) and (*f*)). The mean chloroplast number of WS mesophyll cells is 83, compared to the Ler mean of 120 chloroplasts per mesophyll cell (Table 3.1). The WS mesophyll cell chloroplasts are slightly larger than Ler chloroplasts (mean chloroplast plan area of 52.3 μm^2 in WS, 50.3 μm^2 in Ler, Table 3.1) and the WS mesophyll cell slightly smaller than Ler (WS mean cell plan area 4204 μm^2, Ler mean of 4778 μm^2). The mean total chloroplast cover in WS is therefore 72.69% that of Ler. This difference between the wild type phenotypes of WS and Landsberg *erecta* was accounted for in the comparison of the *arc* mutant whole plant and mesophyll cell phenotypes.

FIGURE 3.4 Comparison of the Ler and WS ecotypes

Mature (7 week old) plants (*a,b*); 21 day old seedlings (*c,d*) and isolated mesophyll cells (*e,f*) of *Arabidopsis thaliana* ecotypes Landsberg *erecta* and WS. Bar = (*a, b*) 50mm; (*c, d*) 10mm; (*e, f*) 25μm. Note the difference in scale between figures *(a)* and *(b)*.

Figure 3.4

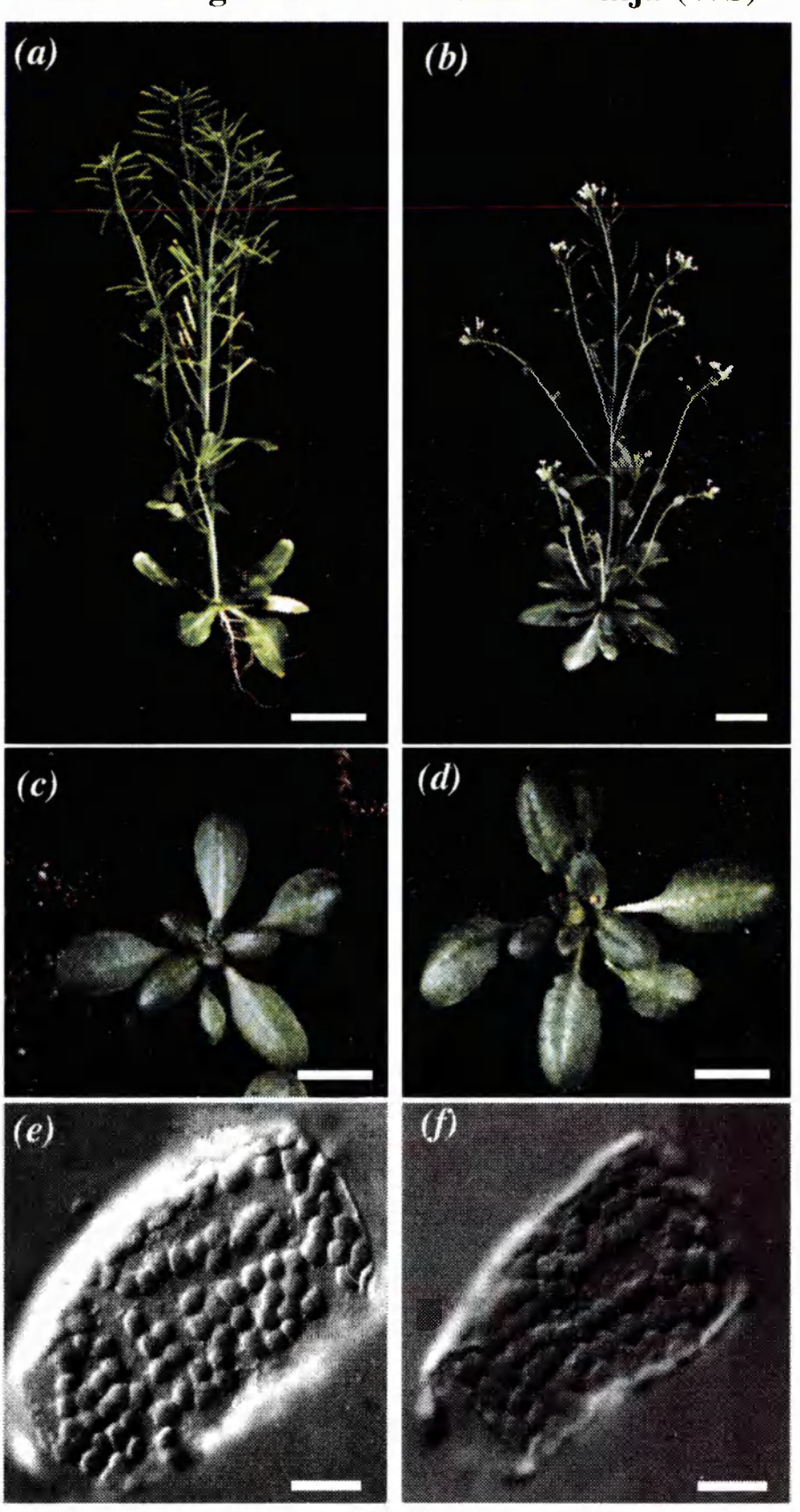


Table 3.1 Mean data for *arc* mutant phenotypes

Mean mesophyll cell size, chloroplast number and chloroplasts size for populations of fully expanded mesophyll cells from first leaves of Landsberg *erecta* and WS wild type and *arc* mutants *arc*1 to *arc*11 of *Arabidopsis thaliana*. Measurements taken from isolated first leaf mesophyll cells viewed with Nomarski differential contrast optics. Standard errors are shown in brackets.

	Mean Mesophyll Cell plan area (μm^2)	Chloroplasts per mesophyll cell	Mean chloroplast plan area (μm^2)	Chloroplasts per 1000μm^2 mesophyll cell plan area
L.*er* wild type	4778 (135)	121	50.3 (0.5)	25
***arc*1**	3388 (86)	108	25.8 (0.3)	32
arc2	4339 (106)	40	111.3 (3.9)	9
***arc*3**	3582 (56)	18	202.1 (9.3)	5
***arc*5**	3531 (135)	13	312 (11)	4
***arc*11**	4732 (531.2)	33	113.2 (9.3)	7
WS wild Type	4204 (150.9)	83	52.3 (0.7)	23
***arc*6**	2077 (158.7)	2.5	650 (58.3)	1
arc7	3384 (93.1)	82	40.4 (0.5)	26
***arc*8**	4381 (398.2)	45	109 (4.3)	10
***arc*9**	2884 (201.7)	34	143.8 (9.4)	12
***arc*10**	4946 (206.3)	38	168 (11.3)	8

3.2.2 The arc *mutants isolated from mutagenised populations of* Arabidopsis

A comprehensive list summarising the origins and phenotypes of both EMS, T-DNA and transposon-mutagenised *arc* mutant phenotypes is summarised in tables 3.1 and 3.2. A summary is also available in figure **A** at the beginning of this thesis.

The screen of the first 9 000 seedlings from the T-DNA mutagenised populations, from which *arc*6-1, *arc*7,*arc*8, *arc*9 and *arc*10 were isolated, was performed jointly with Dr K. Pyke. The further screen of 4 000 T-DNA mutagenised seedlings (a total of 13 000 seedlings screened), from which *arc*6-2 was isolated, and the screening of the Δ*Nae*I-*Ac* -mutagenised populations, from which *arc*11 was isolated, was performed by Dr Pyke.

Individual seedlings from the two insertional mutagenised populations of *Arabidopsis thaliana* were screened microscopically by eye for significant *arc* mutant phenotypes in the mesophyll cells of the first leaf. Fourteen putative mutant individuals were isolated from different pools of T-DNA mutagenised seed. Selfed F_1 progeny of these individuals was re-sown and analysed for a stable *arc* mutant chloroplast phenotype. Six of the putative mutants displayed significant alterations to chloroplast number and size per mesophyll cell in the first leaves of F_1 generation seedlings. Subsequent allelic crossing between mutant individuals demonstrated allelism between two of the mutants (later termed *arc*6-1 and *arc*6-2). The screen of the transposon-mutagenised populations yielded one family of seedlings containing mutant individuals.

The T-DNA and transposon-mutagenised *arc* mutants show several similarities in the development of the chloroplast complement to the published EMS-mutagenised mutants whose characteristics have already been described (Pyke and Leech, 1992, 1994). The data for the EMS-mutagenised *arc* mutant phenotypes is therefore presented here for comparison with the T-DNA and transposon-mutagenised *arc* mutants. The cellular phenotype of typical first leaf mesophyll cells of Landsberg *erecta* wild type and *arc*1, *arc*2, *arc*3, *arc*5 and *arc*11 mutants are illustrated in figure 3.5. The mutant cellular phenotype of typical first leaf mesophyll cells of WS wild type and *arc*6, *arc*7, *arc*8, *arc*9 and *arc*10 mutants are illustrated in figure 3.6. The cells illustrated in these figures are representative of a large number of cells which have been observed in each mutant.

Table 3.2 Summary of *arc* mutant phenotypes

MUTANT ALLELE	POPULATION (Ecotype)(Mutagen)	CHLOROPLAST MUTANT PHENOTYPE IN FIRST LEAF MESOPHYLL CELLS	PLANT MUTANT PHENOTYPE
***arc*1**	Ler EMS	More chloroplasts which are half the size of WT	Young seedling is pale in early development
***arc*2**	Ler EMS	Slightly fewer chloroplasts than wild type; chloroplast size variable	No mutant whole plant phenotype
***arc*3**	Ler EMS	No evidence of chloroplast division. Very few, large, unusually shaped chloroplasts.	No mutant whole plant phenotype
***arc*4**	Ler EMS	Slightly fewer chloroplasts than wild type, chloroplasts moderately varied in size	No mutant whole plant phenotype
***arc*5**	Ler EMS	Very few large chloroplasts which are centrally. constricted. No increase in chloroplast number.	No mutant whole plant phenotype
***arc*6-1**	WS T-DNA N2646	Between 1-4 very large chloroplasts. Plastids cover inner surface of cell in a monolayer	Primary leaves are slightly twisted. Mesophyll cells display distorted shape.
***arc*6-2**	WS T-DNA N2606	Between 1-4 very large chloroplasts. Plastids cover inner surface of cell in a monolayer	Primary leaves are severely twisted. Plant growth very stunted. Distorted mesophyll cell shape
***arc*7**	WS T-DNA N2612	More chloroplasts which are smaller than WT	Leaves of seedling are pale in early development
***arc*8**	WS T-DNA N2619	Slightly fewer chloroplasts than wild type.	No mutant whole plant phenotype.
***arc*9**	WS T-DNA N2624	Most chloroplasts are larger than wild type, *c*.2 chloroplasts per cell are very large	Plant is slightly dwarfed.
***arc*10**	WS T-DNA N2629	Most chloroplasts are slightly larger than wild type. *c*.2 plastids per cell are very large	No mutant whole plant phenotype
***arc*11**	Ler Transposon 02213-3(FG 122)	Fewer chloroplasts than wild type. Considerable variation in chloroplast size.	No mutant whole plant phenotype

FIGURE 3.5 Ler wild type and *arc* mutant mesophyll cells

Photomicrograph of isolated mesophyll cells from fully expanded first leaves of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype Landsberg *erecta* viewed with Nomarski differential contrast optics. (*a*) Landsberg *erecta* wild type; (*b*) *arc*1; (*c*) *arc*2; (*d*) *arc*3; (*e*) *arc*5; (*f*) *arc*11. Bar = 25μm.

Figure 3.5

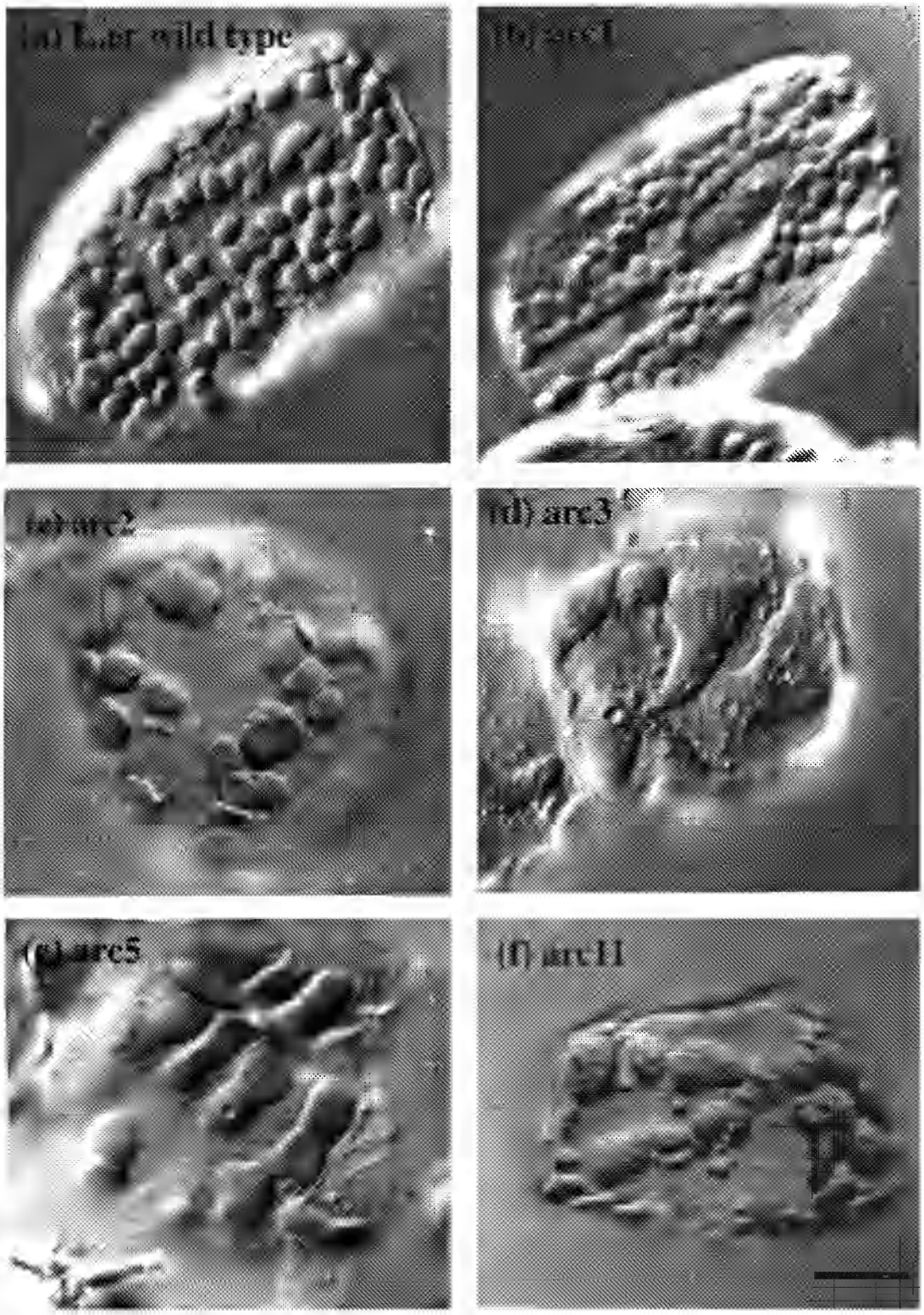
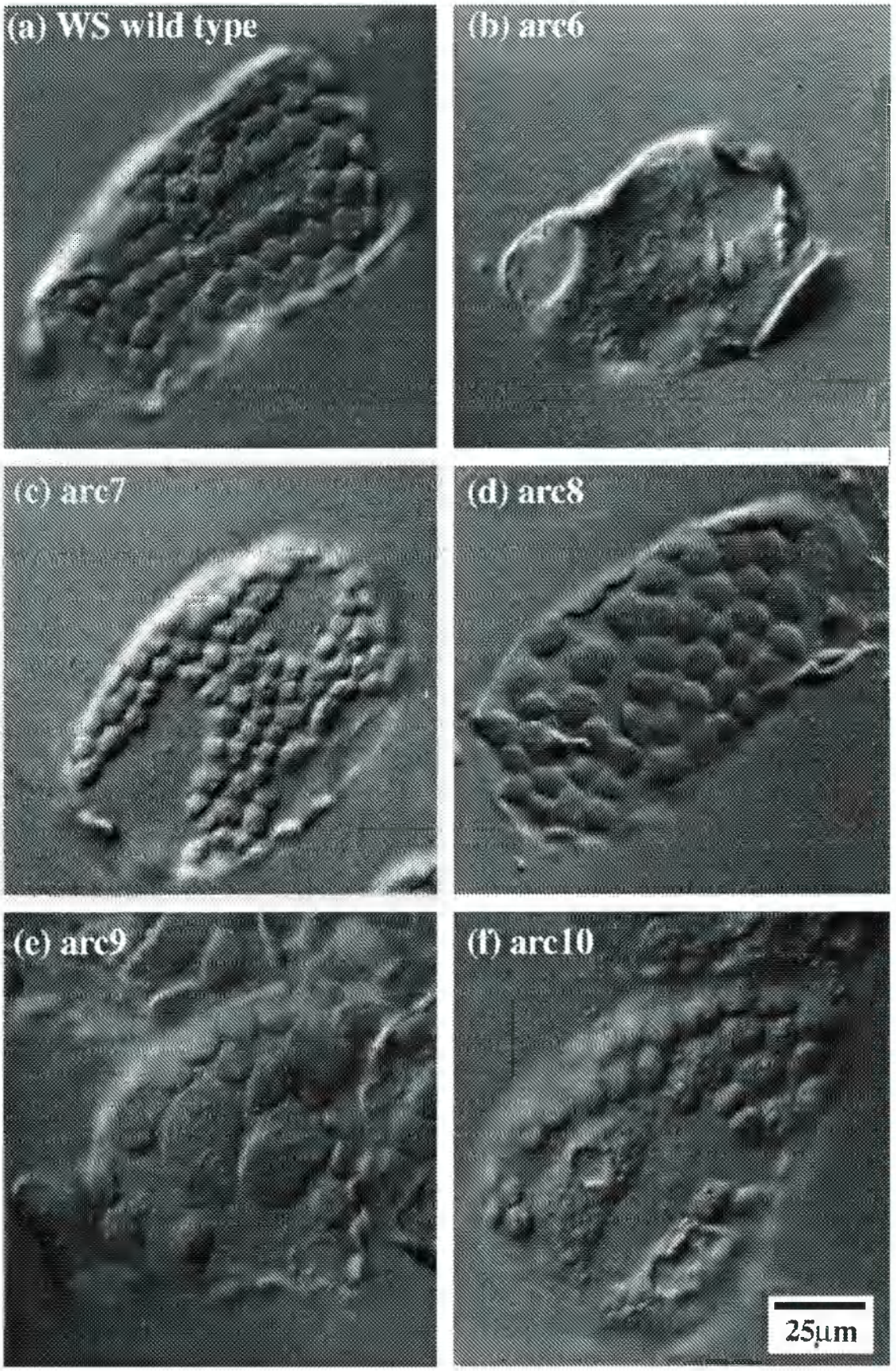


FIGURE 3.6 WS wild type and *arc* mutant mesophyll cells

Photomicrograph of isolated mesophyll cells from fully expanded first leaves of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype WS viewed with Nomarski differential contrast optics. (*a*) WS wild type; (*b*) *arc*6-1; (*c*) *arc*7; (*d*) *arc*8; (*e*) *arc*9; (*f*) *arc*10. Bar = 25μm.

Figure 3.6



The mesophyll cell phenotypes of the mutants were analysed to show the change in chloroplast number and size in relation to increasing cell size in individual mesophyll cells of the first leaf. The detailed results of the analysis of each individual mutant phenotype is discussed later, however the *arc* mutants share several similarities in the development of chloroplast number and size, so general observations may also be drawn.

(i) Chloroplast number: All of the mutants which increased in chloroplast number during mesophyll cell expansion showed a close relationship between chloroplast number and mesophyll cell size (figure 3.7 and 3.8). This relationship was also noted in the mesophyll cells of both Landsberg *erecta* and WS wild type, and was consistently observed in all previously described *arc* mutants (Pyke and Leech, 1992, 1994). The close relationship of increasing chloroplast number and cell size is of considerable importance to the control of chloroplast accumulation in *Arabidopsis* and is discussed later. The *arc* mutants represent a range of means of between 2 and 120 chloroplasts per mesophyll cell and include phenotypes which accumulate more chloroplasts per unit of cell area than wild type as well as mutants which do not increase in chloroplast number at all during mesophyll cell development.

(ii) Chloroplast size: The distribution of chloroplast plan areas in fully expanded first leaf mesophyll cells was also investigated and is shown in figures 3.9 and 3.10. The *arc* mutants demonstrated a variety of chloroplast plan areas over a 100 fold range from half the size of wild type to 50 fold larger in mesophyll cells of the first leaf.

(iii) Total chloroplast area: The accumulation of the total photosynthetic complement of chloroplasts per mesophyll cell was also analysed in relation to mesophyll cell expansion. The proportion of the mesophyll cell surface covered by the chloroplast complement was observed to be consistent between all novel *arc* mutants and wild type during mesophyll cell expansion (figures 3.11 and 3.12). The constancy in total chloroplast area per cell despite a 60 fold variation in chloroplast number and 100 fold variation in chloroplast size per cell illustrates the very high degree of plasticity which is evident in the development of the chloroplast complement in *Arabidopsis*. The uniform total chloroplast area per cell is due to a compensation between chloroplast number and chloroplast size. In mesophyll cells with more chloroplasts than wild type the chloroplasts

FIGURE 3.7 Increasing chloroplast number and cell size in Ler wild type and *arc* mutants

The relationship between chloroplast number per mesophyll cell and mesophyll cell plan area for wild type (dashed line) and *arc* mutants (solid line) of *Arabidopsis thaliana*, ecotype Landsberg *erecta*. Each data point represents the measurement from one cell. The dotted line in figures (*b*) to (*f*) represents the regression line of wild type. Values of r^2 are (*a*) 0.94 for Landsberg *erecta* wild type; (*b*) 0.90 for *arc*1; (*c*) 0.46 for *arc*2; (*d*) 0.17 for *arc*3; (*e*) 0.08 for *arc*5; (*f*) 0.839 for *arc*11. Data for *arc*1, *arc*2 and *arc*3 from Pyke and Leech (1992).

Figure 3.7

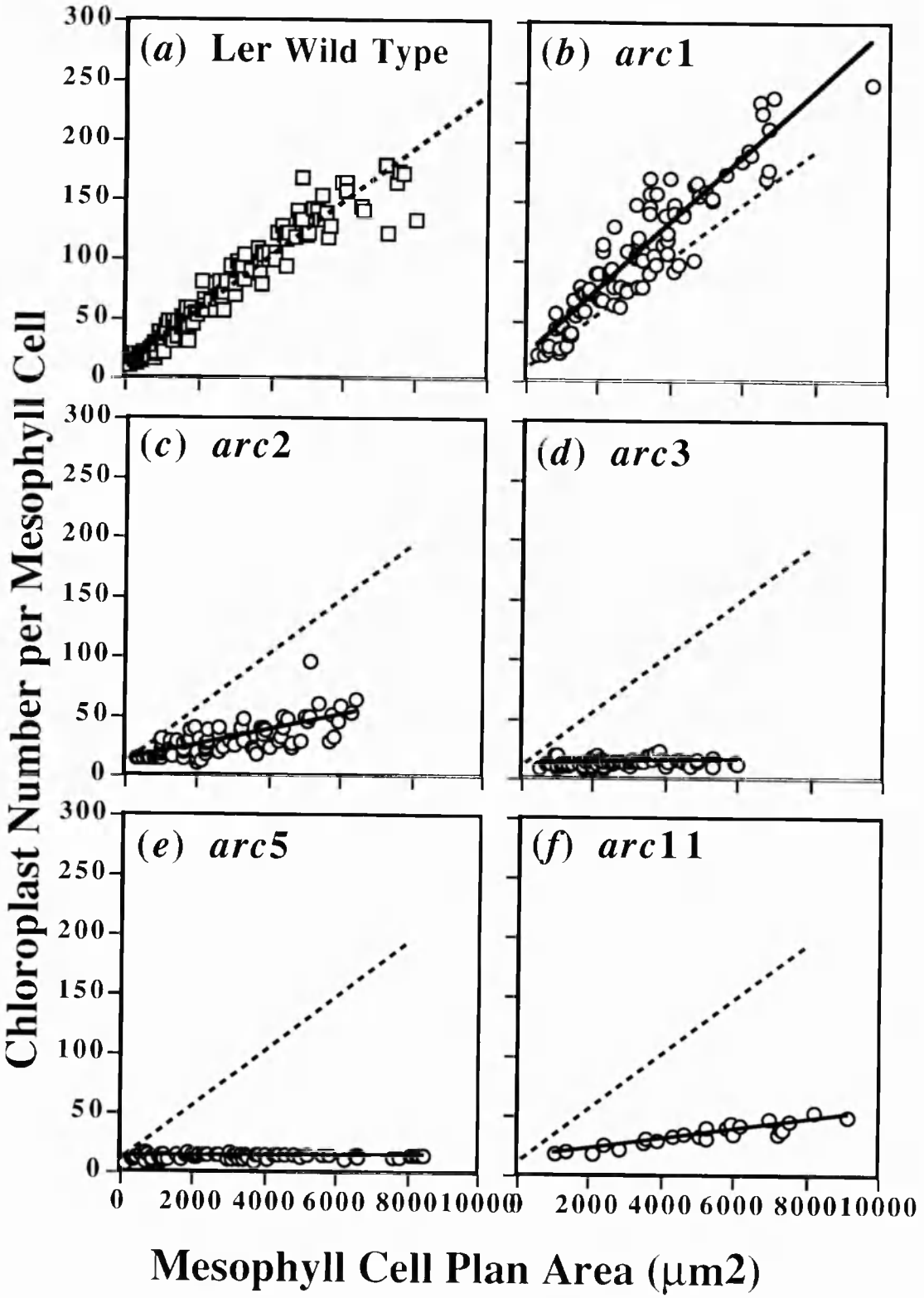

(a) Ler Wild Type
(b) arc1
(c) arc2
(d) arc3
(e) arc5
(f) arc11
Chloroplast Number per Mesophyll Cell
Mesophyll Cell Plan Area (μm2)
0
50
100
150
200
250
300
2000
4000
6000
8000
10000

FIGURE 3.8 Increasing chloroplast number and cell size in WS wild type and *arc* mutants

The relationship between chloroplast number per mesophyll cell and mesophyll cell plan area for wild type (dashed line) and *arc* mutants (solid line) of *Arabidopsis thaliana*, ecotype WS. Each data point represents the measurement from one cell. The linear regression is represented as a bold line. The dotted line in figures (*b*) to (*f*) represents the regression line of wild type. Values of r^2 are (*a*) 0.865 for WS wild type; (*b*) 0.299 for *arc*6; (*c*) 0.680 for *arc*7; (*d*) 0.717 for *arc*8; (*e*) 0.582 for *arc*9; (*f*) 0.171 for *arc*10.

Figure 3.8

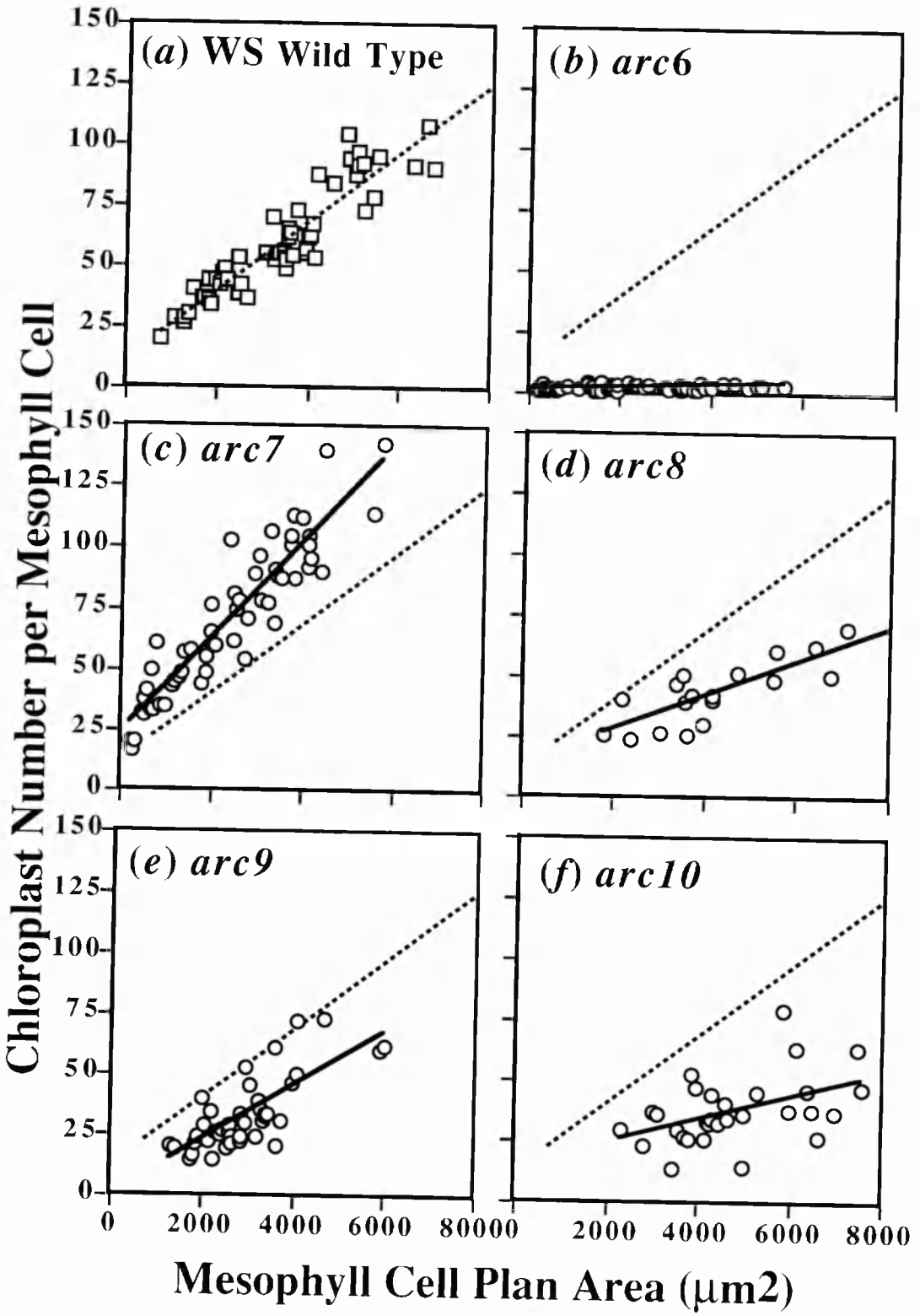

(a) WS Wild Type
(b) arc6
(c) arc7
(d) arc8
(e) arc9
(f) arc10
Chloroplast Number per Mesophyll Cell
Mesophyll Cell Plan Area (μm2)
0
25
50
75
100
125
150
2000
4000
6000
8000
80000

FIGURE 3.9 Chloroplast size distributions in Ler wild type and *arc* mutants

Frequency distributions of chloroplast plan area for mesophyll cell chloroplasts of fully expanded first leaves of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype Landsberg *erecta* . (*a*) Landsberg *erecta* wild type; (*b*) *arc*1; (*c*) *arc*2; (*d*) *arc*3; (*e*) *arc*5; (*f*) *arc*11.

Figure 3.9

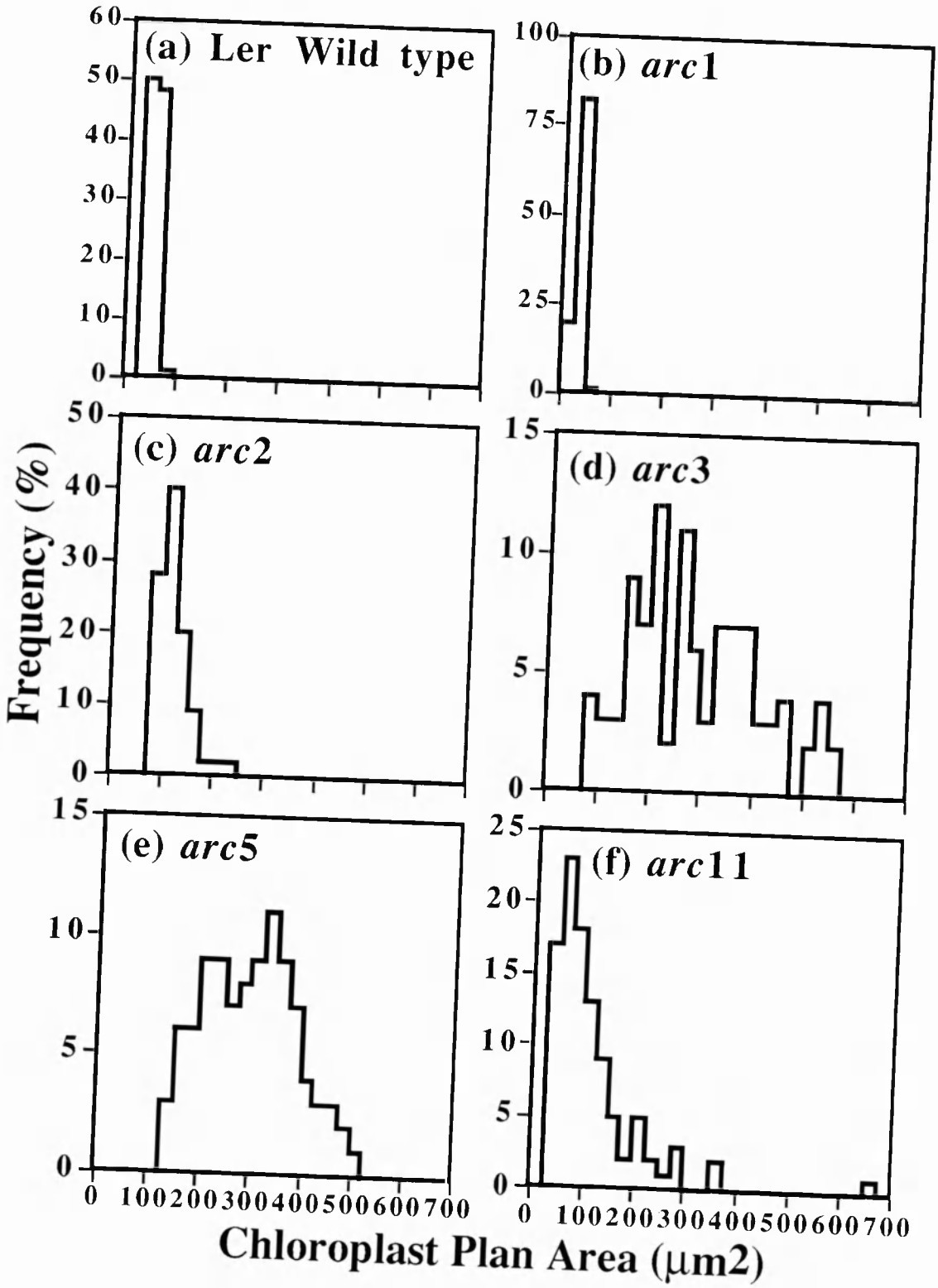

(a) Ler Wild type
(b) arc1
(c) arc2
(d) arc3
(e) arc5
(f) arc11
Frequency (%)
Chloroplast Plan Area (μm2)
0 100 200 300 400 500 600 700

FIGURE 3.10 Chloroplast size distributions in WS wild type and *arc* mutants

Frequency distributions of chloroplast plan area for mesophyll cell chloroplasts of fully expanded first leaves of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype WS. (*a*) WS wild type; (*b*) *arc*6-1; (*c*) *arc*7; (*d*) *arc*8; (*e*) *arc*9; (*f*) *arc*10.

Figure 3.10

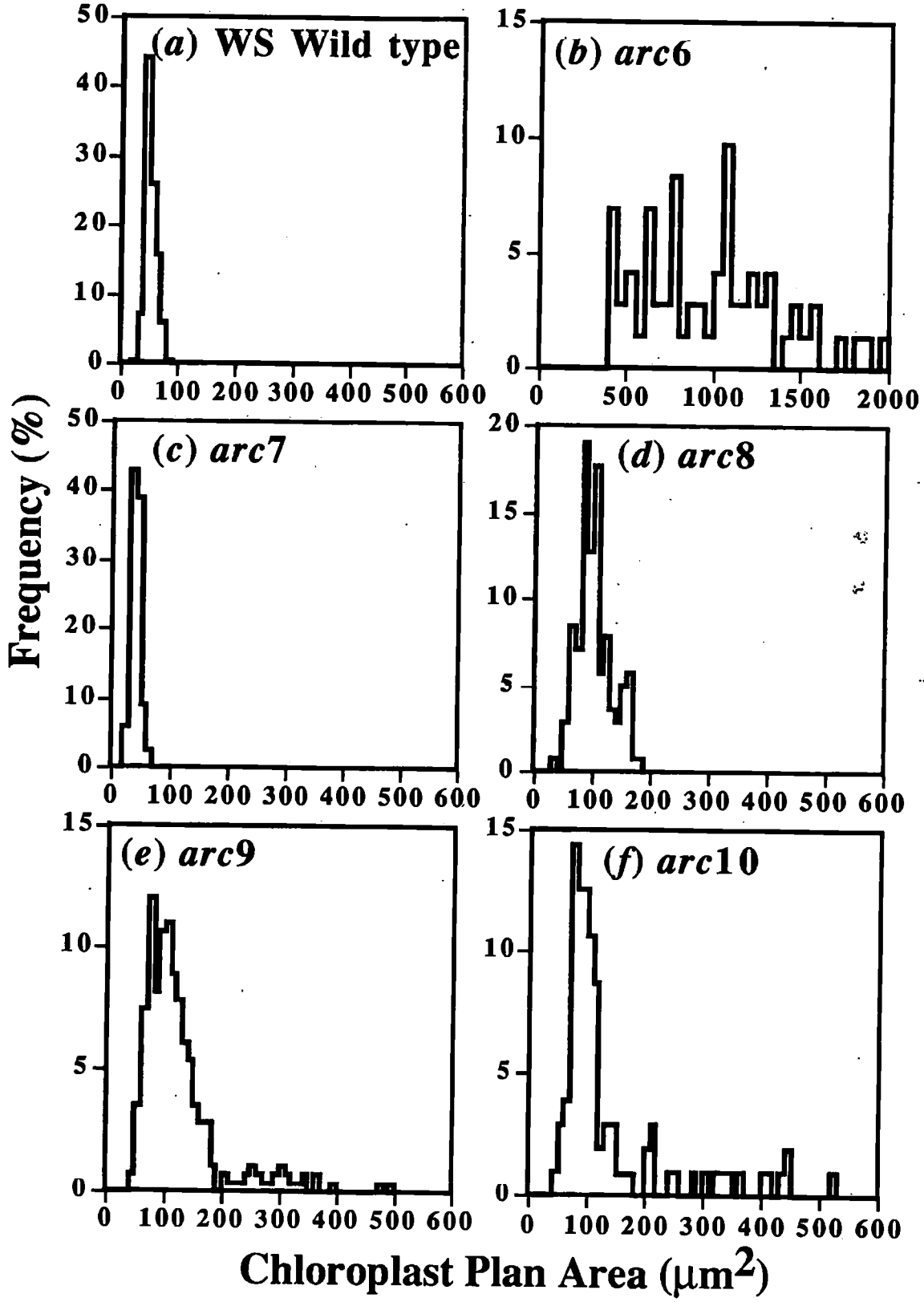

(a) WS Wild type
(b) arc6
(c) arc7
(d) arc8
(e) arc9
(f) arc10
Frequency (%)
Chloroplast Plan Area (μm2)

FIGURE 3.11 Increasing total chloroplast area and cell size in Ler wild type and *arc* mutants

The relationship between total chloroplast plan area per mesophyll cell (determined as the product of mean chloroplast size and chloroplast number) and mesophyll cell plan area for wild type (dashed line) and *arc* mutants (solid line) of *Arabidopsis thaliana*, ecotype Landsberg *erecta*. Each data point represents the measurement from one cell. The dotted line in figures (*b*) to (*f*) represents the regression line of wild type. Values of r^2 are (*a*) 0.93 for Landsberg *erecta* wild type; (*b*) 0.93 for *arc*1; (*c*) 0.81 for *arc*2; (*d*) 0.61 for *arc*3; (*e*) 0.86 for *arc*5; (*f*) 0.87 for *arc*11. Data for *arc*1, *arc*2 and *arc*3 from Pyke and Leech (1992).

Figure 3.11

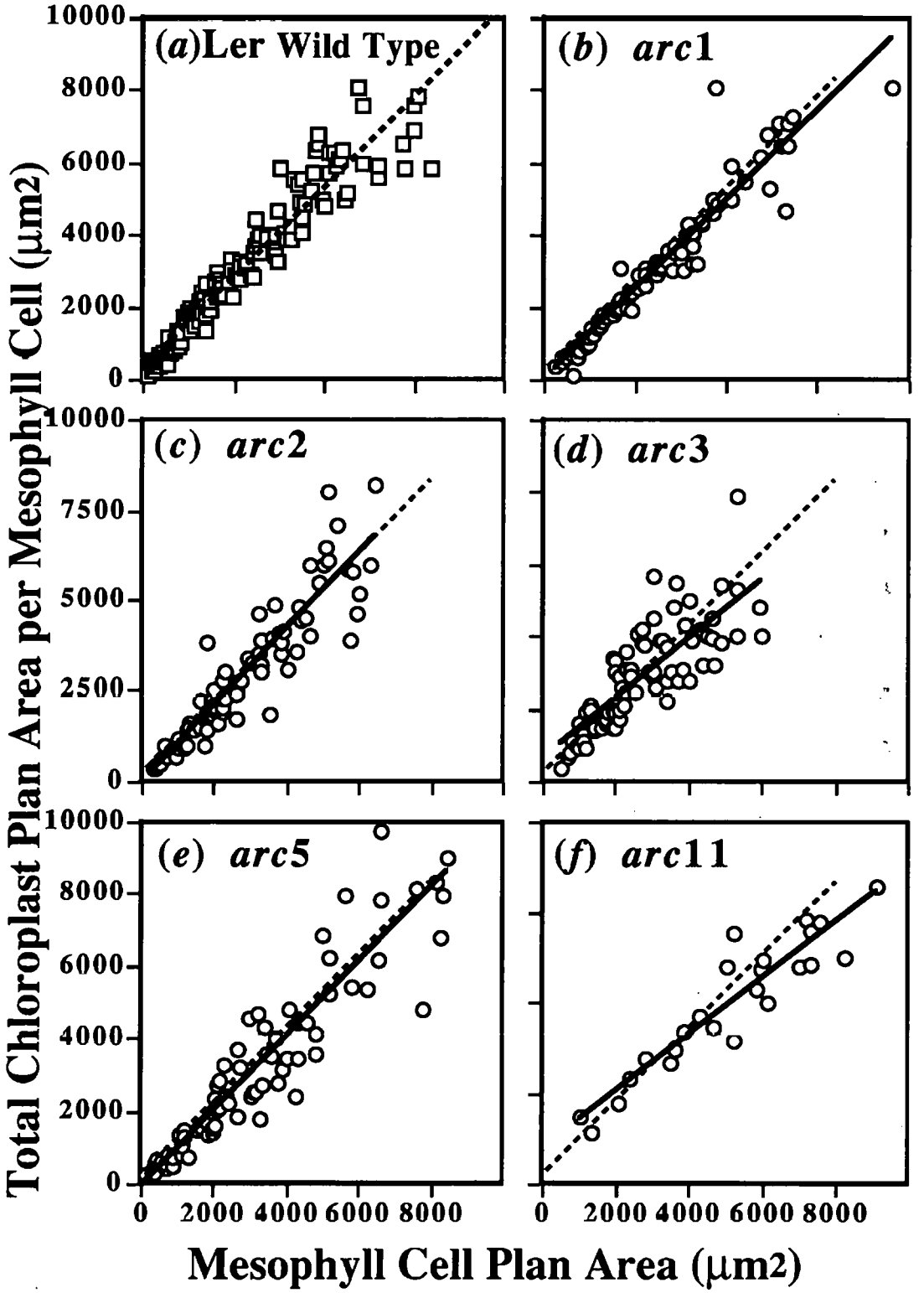
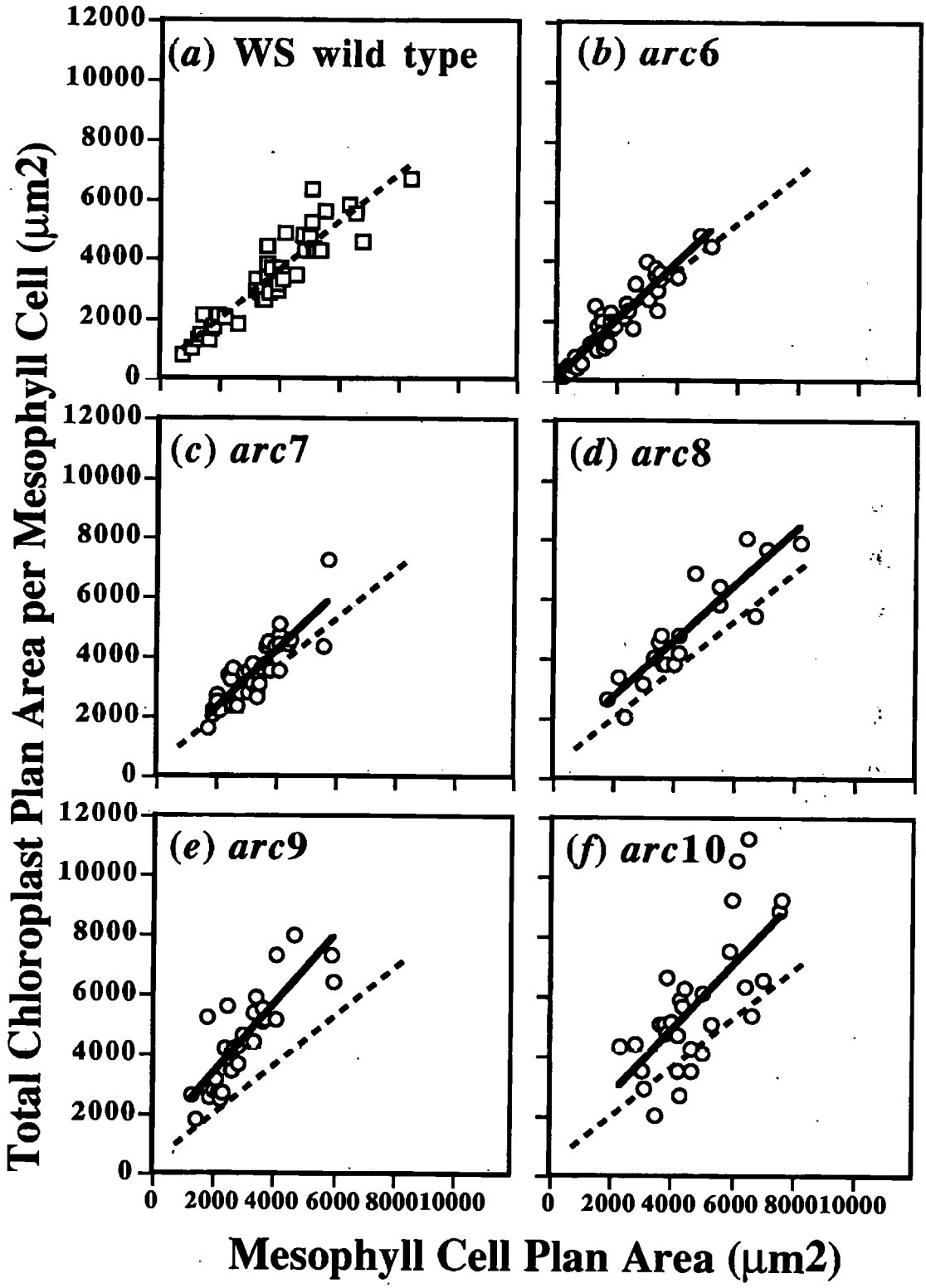

(a)Ler Wild Type
(b) arc1
(c) arc2
(d) arc3
(e) arc5
(f) arc11
Total Chloroplast Plan Area per Mesophyll Cell (μm2)
Mesophyll Cell Plan Area (μm2)
0
2000
4000
6000
8000
10000
2500
5000
7500

FIGURE 3.12 Increasing total chloroplast area and cell size in WS wild type and *arc* mutants

The relationship between total chloroplast plan area per mesophyll cell (determined as the product of mean chloroplast size and chloroplast number) and mesophyll cell plan area for wild type (dashed line) and *arc* mutants (solid line) of *Arabidopsis thaliana*, ecotype WS. Each data point represents the measurement from one cell. The linear regression is represented as a bold line. The dotted line in figures (*b*) to (*f*) represents the regression line of wild type. Values of r^2 are (*a*) 0.85 for WS wild type; (*b*) 0.912 for *arc*6; (*c*) 0.742 for *arc*7; (*d*) 0.815 for *arc*8; (*e*) 0.705 for *arc*9; (*f*) 0.480 for *arc*10.

Figure 3.12


(a) WS wild type
(b) arc6
(c) arc7
(d) arc8
(e) arc9
(f) arc10
Total Chloroplast Plan Area per Mesophyll Cell (µm2)
Mesophyll Cell Plan Area (µm2)
0
2000
4000
6000
8000
10000
12000

are smaller than those of wild type; in those mesophyll cells which do not increase in chloroplast number the chloroplast size is increased to an extreme degree compared to wild type. The compensation of chloroplast number and chloroplast size, maintaining a relatively constant proportion of the cell which is covered by chloroplasts, is likely to be the reason why the majority of *arc* mutants show no significant whole plant mutant phenotype.

(iv) Proplastid number: Another phenotypic feature which is common to all wild type and most of the *arc* mutants is that the smallest mesophyll cells (500-800μm^2, representative of the post-meristematic cells) of the first leaf have a chloroplast number of approximately 14 chloroplasts. This constant number of chloroplasts in the post-meristematic cells of the *Arabidopsis* leaf suggests that the number of immature chloroplasts which have developed from proplastids in the post-meristematic mesophyll cells of the leaf is probably close to 14 (Pyke and Leech, 1991 and 1992). The base number of 14 chloroplasts is noted in all the *arc* mutants except *arc*6 which rarely accumulates more than 3 or 4 chloroplasts per mesophyll cell. This constant number of chloroplasts in the post-meristematic cells suggests that all *arc* mutants except *arc*6 contain the wild type number of proplastids indicating that these *arc* mutants are mutations of chloroplast rather than proplastid division. The *arc*6 mutant is therefore almost certainly a mutant of proplastid division and the base number of 14 chloroplasts is not achieved in the meristematic cells. Chloroplast division is clearly also impaired in the *arc*6 mutant as well as proplastid division since the *arc*6 mutant does not increase its chloroplast number during cell expansion. The observation that some *arc* mutants which are mutants of chloroplast division may be normal for proplastid division shows that the two processes are exclusive with differing genetic controls.

(v) CtDNA content: The observations of K. Pyke (unpublished) on the DNA content of mutant chloroplasts in sectioned mutant leaf tissue stained with the DNA stain DAPI, suggested that CtDNA content per chloroplast in the large mutant chloroplasts was increased in proportion to the size of the plastid. The reduced number of large chloroplasts in *arc*3 and *arc*5 mesophyll cells balanced with an increase in CtDNA content per plastid would ensure a *cellular* CtDNA content similar to that of wild type. An increase

in the CtDNA content of large chloroplasts would imply that, at a cellular level, CtDNA content was constant despite variations in chloroplast number.

In order to compare the ratio between NcDNA and CtDNA amounts contained in the cells *arc* mutants with altered chloroplast number to wild type, a series of Southern slot blots was performed with denatured genomic DNA probed with DNA probes to either the NcDNA or CtDNA . The slotblot filters were probed with a fragment of the 25s rRNA gene of the NcDNA or a fragment of the rbcL gene of the CtDNA. The comparison of the intensity for slots containing 1μg and 2μg of bound, denatured genomic DNA for all *arc* alleles as well as Ler, WS and Columbia wild types is illustrated in figure 3.16 and table 3.3. The results represent preliminary data and require verification by further work. The autoradiographs shown in figure 3.13 are taken from successive hybridisations of the 25s and rbcL probes to the same Southern blot filter. The 25s probe was stripped from the filter after exposure and the stripped filter was re-exposed to verify that no 25s signal remained before hybridisation with the rbcL probe.

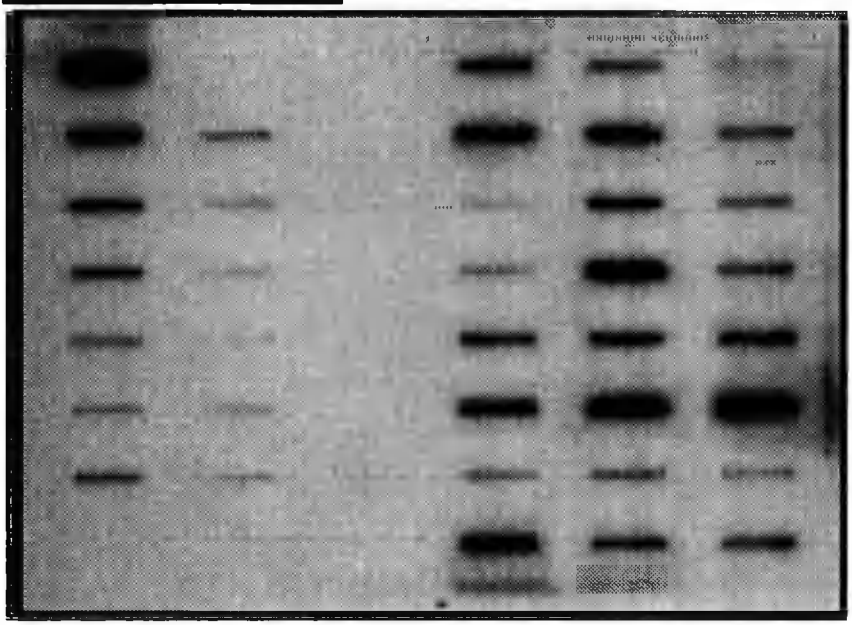
The comparison of the intensity between respective slots in the autoradiographs of NcDNA and CtDNA probes provides a ratio of the amounts of NcDNA to CtDNA per μg of genomic DNA (table 3.3). An increase in this ratio indicates a reduction in the CtDNA amount relative to the NcDNA. This effect was observed to a moderate degree in the *arc*1, *arc*6, *arc*7 and *arc*8 mutants. A slight increase in the CtDNA amount was indicated in the *arc*2, *arc*9 and *arc*10 mutants. *arc*3, *arc*5 and *arc*11 are not significantly different to wild type. None of the *arc* mutants however demonstrated as extreme a deviation from wild type in the ratio of CtDNA to NcDNA amount which was comparable to the wide variation in chloroplast number between the *arc* mutants and wild type. A deviation in the CtDNA to NcDNA ratio between mutant and wild type samples that was several orders of magnitude in size would be predicted in the *arc* mutants with radically altered chloroplast numbers if CtDNA amount was constant in *all* chloroplasts irrespective of chloroplast size. These preliminary data suggest that CtDNA replication is only slightly affected by lesions in chloroplast replication and expansion in *arc* mutants of *Arabidopsis*. These preliminary findings therefore imply that CtDNA replication is not significantly perturbed by either a lack of or an increase in chloroplast division in *Arabidopsis*.

FIGURE 3.13 Comparison of NcDNA and CtDNA amount in wild type and *arc* mutants

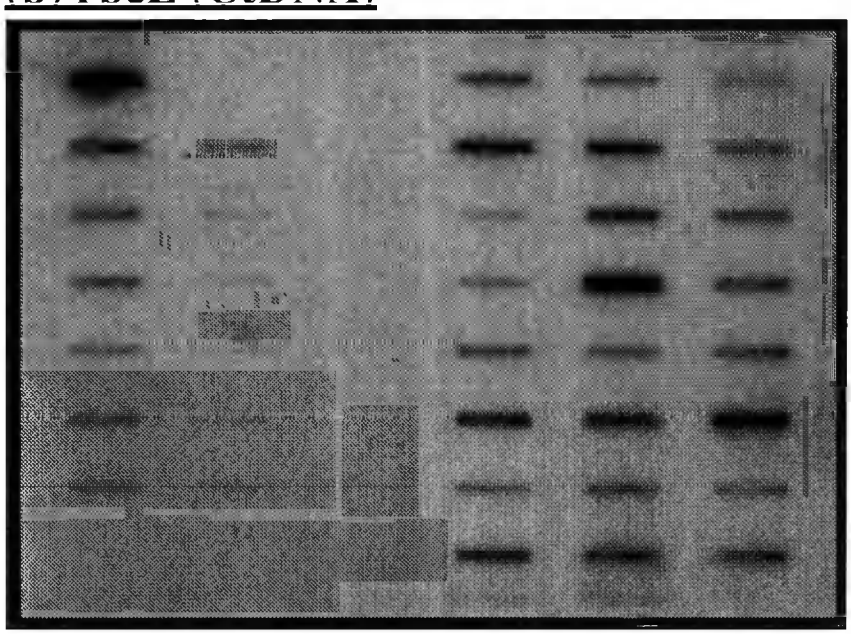
Autoradiographs of a single slot blot of genomic DNA of wild type and *arc* mutants of *Arabidopsis thaliana* hybridised to ^{32}P -labelled fragments of (*a*) 25S (NcDNA) and (*b*) rbcL (CtDNA) genes respectively. The legend indicates the identity and quantity of DNA (μg) of each slot sample. Autoradiograph exposure was for 48 hours. Prior to the hybridisation to the 25S probe, the slot blot filter was stripped of rbcL probe and exposed for 48 hours to verify the lack of any remaining radioactive label.

Figure 3.13

(a) 25s (NcDNA)



(b) rbcL (CtDNA)



L.er 5ug		arc1 1ug	arc6-1 1ug	arc10 1ug
L.er 2.5ug	WS 2.5ug	arc1 2ug	arc6-1 2ug	arc10 2ug
L.er 1ug	WS 1ug	arc2 1ug	arc7 1ug	arc11 1ug
L.er 0.5ug	WS 0.5ug	arc2 2ug	arc7 2ug	arc11 2ug
L.er 0.1ug	WS 0.1ug	arc3 1ug	arc8 1ug	arc6-2 1ug
L.er 75ng	WS 75ng	arc3 2ug	arc8 2ug	arc6-2 2ug
L.er 50ng	WS 50ng	arc5 1ug	arc9 1ug	Col WT 1ug
		arc5 2ug	arc9 2ug	Col WT 2ug

Table 3.3 Relative Nc DNA and CtDNA content of wild type and *arc* mutants

Relative concentrations of nuclear and chloroplast DNA in wild type and *arc* mutants of *Arabidopsis* as revealed by hybridisation of ^{32}P-labelled 25s (NcDNA) or rbcL (CtDNA) probes to slotblot of denatured genomic DNA (figure 3.16). Relative values of absorbence for individual slots were measured by an image analysis system on images of slotblot autoradiographs captured by CCDTV camera. Values of *arc* mutants are means of a 1µg and a 2µg slot of DNA for each *arc* mutant sample (Standard deviations are shown in parentheses). L.*er* wild type value is a mean of 5, 2.5, 1, 0.5, 0.1, 0.075 and 0.005 µg slots; WS wild type value is a mean of 2.5, 1, 0.5, 0.1, 0.075 and 0.005 µg slots. The ratio of NcDNA (25s) to CtDNA (rbcL) is shown suggesting a slight decrease in Ct DNA relative to NcDNA in *arc*1, *arc*6, *arc*7 and *arc*8.

	Absorbance (arbitrary units)		Ratio
***arc* Mutant**	**25s** (NcDNA)	**rcbL** (CtDNA)	**25s / rbcL**
L.*er* WT	0.2141 (0.108)	0.3821 (0.617)	0.560
WS WT	0.1388 (0.066)	0.2389 (0.044)	0.588
***arc*1**	0.2295 (0.049)	0.3800 (0.050)	0.604
***arc*2**	0.1543 (0.028)	0.3147 (0.028)	0.490
***arc*3**	0.1962 (0.036)	0.3717 (0.029)	0.577
***arc*5**	0.2025 (0.077)	0.3739 (0.063)	0.542
***arc*6-1**	0.2364 (0.086)	0.3711 (0.064)	0.637
***arc*6-2**	0.2806 (0.106)	0.3975 (0.069)	0.706
***arc*7**	0.3227 (0.112)	0.4405 (0.039)	0.733
***arc*8**	0.2858 (0.094)	0.3592 (0.051)	0.796
***arc*9**	0.1868 (0.030)	0.3952 (0.048)	0.472
***arc*10**	0.1457 (0.018)	0.3253 (0.024)	0.448
***arc*11**	0.2806 (0.037)	0.3522 (0.499)	0.532

(vi) Whole plant phenotype: The whole plant phenotype of wild type and *arc* mutant seedlings is illustrated in figure 3.14; the phenotype of mature, 5 week old plants is illustrated in figures 3.15 and 3.16. The *arc* mutants previously described by Pyke and Leech (1992, 1994) exhibited no significant mutant whole plant phenotype; this lack of a significant mutant whole plant phenotype was also observed in all of the T-DNA and transposon-mutagenised *arc* mutants with the exception of *arc*6-1 and *arc*6-2. The *arc*6 mutants exhibited a twisted leaf phenotype in the primary leaves of both the seedling and the mature plant, which will aid the future isolation of *arc*6 alleles and is discussed in chapter 5. The lack of any significant whole plant mutant phenotype in most *arc* mutants is surprising when one considers the extreme variation in chloroplast number and size in these mutants, and demonstrates the degree of toleration for variations in the chloroplast complement exhibited by the *Arabidopsis* plant.

3.2.3 The arc *mutant phenotypes*

*(a) arc*6

The screen of the T-DNA population yielded two alleles of *arc*6. Both alleles have a similar chloroplast mutant phenotype of a mean of two chloroplasts, however the *arc*6-2 displays a more extreme whole plant mutant phenotype than the *arc*6-1 allele. The *arc*6 mutant phenotype is discussed in more detail in chapter 5. The analysis of the *arc*6 chloroplast phenotype described below was carried out using the *arc*6-1 allele.

The *arc*6 phenotype is the most extreme *arc* mutant phenotype which has so far been characterised. A typical *arc*6 mesophyll cell is illustrated in figure 3.5 (*b*) and shows a single large chloroplast which is seen to cover most of the visible cell surface. The *arc*6 mutant typically contains only between 1 and 3 chloroplasts per mesophyll cell (WS wild type mean of 80 chloroplasts) which are a mean plan area of 650μm^2 (Figure 3.10 (*b*), Table 3.1) but may be up to 2500μm^2 which is 50-fold larger than wild type chloroplasts. The morphology of *arc*6 chloroplasts is highly irregular within the cell, with lobing of the large chloroplast common. The chloroplasts of the *arc*6 mutant accumulate starch, suggesting normal photosynthetic function of the chloroplasts within the mesophyll cells.

FIGURE 3.14 Seedlings of wild type and *arc* mutants

23 day old seedlings of wild type and *arc* mutants of *Arabidopsis thaliana* ecotypes Landsberg *erecta* (*a* to *f*) and WS (*g* to *l*). (*a*) Landsberg *erecta* wild type; (*b*) *arc*1; (*c*) *arc*2; (*d*) *arc*3; (*e*) *arc*5; (*f*) *arc*11. (*g*) WS wild type; (*h*) *arc*6-1; (*i*) *arc*7; (*j*) *arc*8; (*k*) *arc*9; (*l*) *arc*10. Bar = 10mm.

Figure 3.14

FIGURE 3.15 Mature plants of Ler wild type and *arc* mutants

35 day old plants of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype Landsberg *erecta*. (*a*) Landsberg *erecta* wild type; (*b*) *arc*1; (*c*) *arc*2; (*d*) *arc*3; (*e*) *arc*5; (*f*) *arc*11. Bar = 50mm.

Figure 3.15

FIGURE 3.16 Mature plants of WS wild type and *arc* mutants

35 day old plants of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype WS. (*a*) WS wild type; (*b*) *arc*6-1; (*c*) *arc*7; (*d*) *arc*8; (*e*) *arc*9; (*f*) *arc*10. Bar = 50mm.

Figure 3.16

The *arc*6 mutant also displays the most radical whole plant mutant phenotype yet noted in the *arc* mutants. The cotyledons of *arc*6 seedlings undergo early senescence and are very slightly paler than wild type cotyledons. The rosette leaves of *arc*6 exhibit a wrinkled appearance (figure 3.14(*h*), figure 3.16 (*b*), also figure 5.6 and 5.7 in chapter 5) with a transverse buckling of the lamina in an oblique plane to the midvein of the leaf. The *arc*6 mutant appears to flower earlier than wild type by approximately 4-5 days. The *arc*6 plant is able to self-fertilise and set seed normally; however the germination frequency of *arc*6 seed is approximately 80% of wild type under growth room conditions (figure 5.9).

*(b) arc*7

The *arc*7 chloroplasts are of uniform size, approximately 80% of the size of WS chloroplasts (Figure 3.6 *(c)*; Table 3.1). The *arc*7 mutant exhibits a mesophyll cell chloroplast density (per 1000μm^2 cell area) which is 13.4% greater than WS wild type. The mean cell plan area of *arc*7 mesophyll cells is 12.1% less than for WS wild type (Table 3.1) suggesting a reduction in mesophyll cell expansion during *arc*7 development. The reduction in mean cell plan area and the slight reduction of mean chloroplast number per mesophyll cell causes an increased density of 26 chloroplasts per 1000μm^2 of mesophyll cell area compared to the wild type density of 23 chloroplasts (Table 3.1).

The seedlings of *arc*7 display a mutant phenotype of slightly pale leaves in early seedling development. As the seedling develops the leaves become greener so that by approximately 25 days post-germination the seedling is not significantly paler than wild type. The cotyledons of the *arc*7 mutant are green, however, compared to the *arc*1 mutant which has both pale cotyledons and pale leaves.

The *arc*7 plant is slightly reduced in growth rate compared to wild type, flowering *c*.7 days later than wild type. The plant is fully fertile and produces seed normally.

*(c) arc*8

The *arc*8 mutant mesophyll cell phenotype is illustrated in figure 3.6(*d*). The chloroplasts of *arc*8 are approximately twice the size of wild type and chloroplast number is approximately half the wild type number (Table 3.1). The *arc*8 chloroplasts are relatively uniform in size (Figure 3.10 (*d*)), varying from 50 to 200μm^2 and they do not

appear to be different in shape from wild type chloroplasts. The moderate *arc* mutant phenotype of *arc*8 compared to other *arc* mutants suggests that the *arc*8 mutation is not an extreme lesion in the process of chloroplast division so that division may proceed at a reduced rate during mesophyll cell expansion.

There is no mutant whole plant phenotype visible, in either *arc*8 seedlings or mature plants. *arc*8 plants display normal vigour, growth rate and fertility compared to wild type.

*(d) arc*9

The mesophyll cells of the *arc*9 mutant typically contain two sizes of chloroplast which are easily distinguishable and may be observed in figure 3.6 *(e)*. *arc*9 mesophyll cells have a mean number of 34 chloroplasts. *arc*9 mesophyll cells usually contain two chloroplasts which are between 4 and 10 fold larger than wild type chloroplasts. The majority of *arc*9 chloroplasts, however, are smaller and less variable in size with a mean plan area of 110μm^2 which is twice the plan area of WS chloroplasts.

The mature *arc*9 mutant plant is more compact than the normal plant of the WS ecotype (figure 3.16 *(e)*), however this mutant phenotype is not sufficiently characteristic to be distinguishable from WS wild type when the two genotypes are grown together. The *arc*9 mutant is fully fertile and is similar to wild type in its flowering time.

*(e) arc*10

The mutant phenotype of *arc*10 displays similar characteristics to that of *arc*9. Like *arc*9 mesophyll cells, the cells of *arc*10 contain two distinct sizes of chloroplasts (Figure 3.6 *(f)*). The large chloroplasts of *arc*10 are between 4 and 10 fold larger than WS; the smaller chloroplasts are smaller than their equivalent in *arc*9, being only a mean of 90μm^2, varying between wild type size and 3.5 fold larger.

The *arc*10 mutant has no discernible whole plant mutant phenotype and is not significantly different to wild type in developmental rate, vigour or fertility.

*(f) arc*11

The *arc*11 mutant was isolated from T_4 family number 122 of the 02213-3 population of Δ*Nae*I *Ac* -mutagenised *Arabidopsis*. Twelve individual seedlings were

screened from the family, of which two were mutant, two heterozygote wild type (i.e. segregated for wild type and mutant in the next generation) and eight homozygous wild type. The phenotype of *arc*11 is also discussed in detail in sections 6.3.1 to 6.3.3.

The *arc*11 mutant chloroplast phenotype is very variable both between cells and within a single cell (Figure 3.5 (*f*)). The mean chloroplast number for *arc*11 chloroplasts is 33 chloroplasts per mesophyll cell, which is 25% of the Landsberg *erecta* wild type mean (table 3.1). The size of *arc*11 chloroplasts varies between 25 and 400μm^2 (Ler mean of 50μm^2), although larger chloroplasts are also infrequently observed of sizes up to 700μm^2 (figure 3.9 (*f*)). Chloroplast shape is irregular in the population of the larger *arc*11 chloroplasts, less so in the smaller plastids. The highly variable phenotype of *arc*11 is similar to that of *arc*2 (Pyke and Leech, 1992), however the range of the variation in chloroplast number and size within and between mesophyll cells appears to be more extreme in *arc*11 than is observed in *arc*2.

The *arc*11 mutant appears to have no significant mutant whole plant phenotype either in the seedling or the mature plant. The mutant is not reduced in vigour or fertility compared to Landsberg *erecta* wild type.

3.3.4 The genetic analysis of arc *mutants*

All *arc* mutant alleles have been demonstrated to be stably inherited over at least 5 generations (data not shown). The *arc*11 mutant, however, has demonstrated evidence of reversion of the mutant chloroplast phenotype to that of wild type in 1.8% of mutant progeny due to the excision of the *Ac* element (detailed in chapter 6).

Mendelian inheritance of the *arc* mutants was investigated to verify that the *arc* mutants were recessive mutations of nuclear genes. Mendelian genetic characteristics were analysed by backcrossing *arc* mutants to wild type and the analysis of the segregation of resultant progeny. The mutants were backcrossed to a wild type of a different ecotype in order to facilitate subsequent RFLP mapping strategies. The T-DNA induced mutants in the WS ecotype background were backcrossed to Landsberg *erecta* wild type; the transposon-induced mutant *arc*11 of the Ler background was backcrossed to Columbia

wild type. Backcrosses were carried out reciprocally to verify that the *arc* mutants were nuclear mutations, since a mutation which demonstrated maternal inheritance would be likely to be a mutation of the CtDNA rather than the nuclear DNA. The reciprocal backcrosses produced similar segregation ratios which indicated that the mutations were all nuclear mutations rather than maternally inherited mutations of the cytoplasmic genomes. All *arc* mutants exhibited a wild type phenotype in the heterozygous plants of the F_1 generation, indicating that each *arc* mutation was a recessive allele. The progeny of self-pollinated F_1 individuals from the backcrosses segregated 3:1 wild type : mutant chloroplast phenotype (table 3.4), indicating Mendelian inheritance of a recessive allele.

In order to determine if any of the *arc* mutants were allelic, i.e. mutations of the same *ARC* locus, a series of hybrid crosses between mutants was undertaken and the chloroplast phenotype of the F_1 progeny examined. Non-allelic *arc* mutants were predicted to exhibit a wild type chloroplast phenotype in the F_1 generation due to complementation of the two recessive mutations restoring a wild type phenotype. The results of these crosses are summarised in table 3.5. Complementation of the mutant phenotype was noted between two mutants, subsequently named *arc*6-1 and *arc*6-2. All other mutants exhibited a wild type phenotype in the F_1 progeny of the outcross, indicating they were non-allelic.

The outcrosses between mutants were not performed reciprocally since the mutants had already demonstrated to be autosomal nuclear mutations. The outcrosses involving *arc*11 were carried out reciprocally to reduce the risk of a possible false negative result due to an excision of the *Ac* restoring a wild type phenotype in the germline of the *arc*11 plant.

3.2.5 Analysis of the cosegregation of the T-DNA with the arc *mutant loci*

The 1'NPTII gene of the T-DNA conveys resistance of the plant to kanamycin and was used as a marker for the presence of the T-DNA in seedlings. Typical kanR and kan S seedlings grown on 50ng/ml kanamycin medium are shown in figure 3.17 (*a*). The *arc*6, *arc*7, *arc*9 and *arc*10 seedlings were all kanR (table 3.6), suggesting the presence of a T-DNA in the DNA of these mutants. *arc*6-2 and *arc*8 seedlings were kanS, indicating the lack of a functional T-DNA.

Table 3.4

Results of backcrosses of *arc* mutants to wild type

The mendelian characteristics of nuclear recessive alleles are revealed in the *arc* mutants of *Arabidopsis* (ecotype WS and Landsberg *erecta*) by backcrossing to wild type. Mutants were reciprocally backcrossed to wild type Landsberg *erecta* (*arc*6-1 to *arc*10) or Columbia (*arc*11) and the progeny examined for mesophyll cell chloroplast phenotype in the F_1 and F_2 generations. Mesophyll cell phenotypes were scored by microscopic examination of first leaf tissue from individual F_1 and F_2 seedlings after 23 days. The χ^2 calculation was based on a segregation ratio of one mutant to three wild type individuals.

	Number of plants		
Cross	**F_1 generation**	**F_2 generation**	
Female x Male	WT : Mutant	WT : Mutant	Total
*arc*6-1 x L.*er*	19 : 0	207 : 56	263
L.*er* x *arc*6-1	33 : 0	-	-
*arc*6-2 x L.*er*	7 : 0	213 : 54	267
L.*er* x *arc*6-2	24 : 0	-	-
*arc*7 x L.*er*	21 : 0	-	-
L.*er* x *arc*7	18 : 0	208 : 67	275
*arc*8 x L.*er*	23 : 0	200 : 70	270
L.*er* x *arc*8	12 : 0	-	-
*arc*9 x L.*er*	19 : 0	207 : 69	276
L.*er* x *arc*9	17 : 0	-	-
*arc*10 x L.*er*	33 : 0	213 : 57	270
L.*er* x *arc*10	34 : 0	-	-
*arc*11 x Col	24 : 0	245 : 87	332
Col x *arc*11	24 : 0	-	-

Table 3.5

Results of allelic crosses between *arc* mutants

Table of allelic crosses between mutant individuals isolated from mutagenised populations of *Arabidopsis thaliana*. Allelic crosses are represented by: **MUT** = F_1 progeny of allelic cross show fully mutant phenotype (i.e. mutants are allelic); **WT** = F_1 progeny of allelic cross show fully wild type phenotype (i.e. mutants are *not* allelic), allelic cross was carried out by myself; $\mathbf{WT}_{(A)}$ = F_1 progeny of allelic cross show fully wild type phenotype, allelic cross carried out by Pyke and Leech, 1992; $\mathbf{WT}_{(B)}$ = F_1 progeny of allelic cross show fully wild type phenotype, allelic cross carried out by Pyke and Leech, 1994; $\mathbf{WT}_{(C)}$ = F_1 progeny of allelic cross show fully wild type phenotype, allelic cross was carried out by K.A. Pyke (personal communication); **N**=not tested; x =not tested since the reciprocal half of the cross was tested instead.

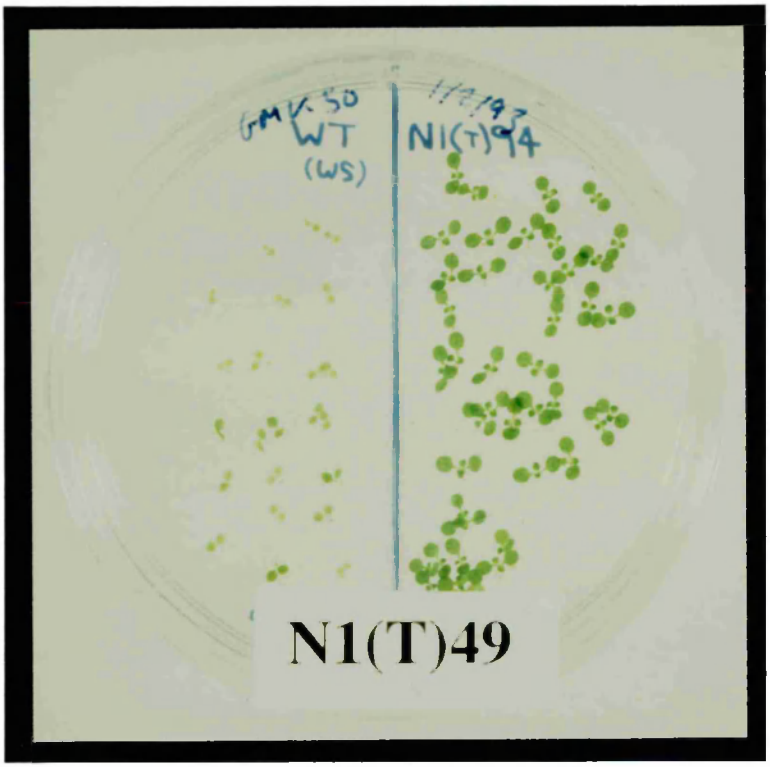
Female / Male	*arc*1	*arc*2	*arc*3	*arc*5	*arc*6-1	*arc*6-2	*arc*7	*arc*8	*arc*9	*arc*10	*arc*11
*arc*1		$WT_{(A)}$	$WT_{(A)}$	$WT_{(B)}$	x	N	$WT_{(C)}$	N	WT	x	WT
*arc*2	$WT_{(A)}$		$WT_{(A)}$	$WT_{(B)}$	N	N	N	x	N	x	WT
*arc*3	$WT_{(A)}$	$WT_{(A)}$		$WT_{(B)}$	WT	N	N	x	x	WT	WT
*arc*5	$WT_{(B)}$	$WT_{(B)}$	$WT_{(B)}$		WT	N	N	x	x	WT	WT
*arc*6-1	WT	N	x	x		MUT	x	x	N	WT	WT
*arc*6-2	N	N	N	N	MUT		N	N	N	N	N
*arc*7	WT	N	N	N	WT	N		x	WT	N	WT
*arc*8	N	$WT_{(C)}$	$WT_{(C)}$	$WT_{(C)}$	WT	N	WT		WT	N	N
*arc*9	x	N	10:0	12:0	N	N	x	x		N	WT
*arc*10	WT	WT	x	x	x	N	N	N	N		N
*arc*11	WT	WT	WT	WT	WT	N	WT	N	WT	N	

FIGURE 3.17 KanR and kanS *Arabidopsis* seedlings

10 day old seedlings of *Arabidopsis thaliana* grown on nutrient medium plus 50mg/l kanamycin antibiotic to select for kanamycin resistance conferred by the presence of a T-DNA. (*a*) WS wild type (kanS) and *arc7* (kanR) seedlings grown together exhibit the kanS phenotype in comparison to kanR. (*b*) Seedlings of the F2 population of a backcross of *arc7* to wild type exhibit a 3:1 segregation of kanR : kanS, demonstrating the presence of a single T-DNA insert in the *arc7* mutant.

Figure 3.17

(a)



(b)

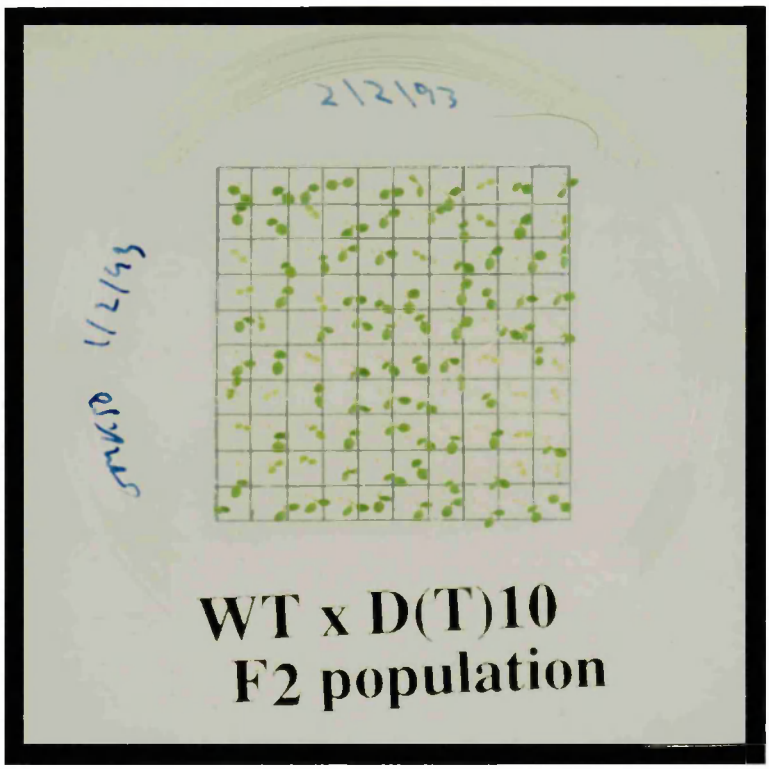


Table 3.6

Segregation of the T-DNA locus with *arc* mutant loci

The presence of a functional T-DNA in the genotype of *arc* mutants of *Arabidopsis* (ecotype WS) is revealed by kanamycin resistance conferred by the T-DNA. *arc* mutants were backcrossed to wild type Landsberg *erecta*. The mutant parents and F_1 and F_2 generation progeny were examined for kanamycin resistance. Plates sown with seed were stored in the dark at 4°C for 48 hours to vernalise seed, kanamycin resistance phenotype was scored 10 days after plates were placed in light conditions. Seed from mutant F_2 siblings grown on soil was tested for kanamycin resistance in the F_3 generation, a fully kanR phenotype indicating linkage of the T-DNA to the mutant locus.

	Number of plants		
	F_1 generation	F_2 generation	F_3 generation
Backcross Female x Male	kanR : kanS	kanR : kanS	kanR : kanS
*arc*6-1 mutant	34 : 0	-	-
*arc*6-1 x L.*er*	99 : 0	297 : 85	211 : 189
*arc*6-2 mutant	0 : 35	-	-
*arc*6-2 x L.*er*	*n/a*	-	-
*arc*7 mutant	41 : 0	-	-
L.*er* x *arc*7	100 : 0	228 : 74	232 : 271
*arc*8 mutant	0 : 43	-	-
*arc*8 x L.*er*	*n/a*	-	-
*arc*9 mutant	24 : 0	-	-
*arc*9 x L.*er*	102 : 0	220 : 80	146 : 155
*arc*10 mutant	40 : 0	-	-
*arc*10 x L.*er*	100 : 0	281 : 17	96 : 103

The segregation ratio of kanR to kanS in the F_2 generation of a backcross of *arc* mutant to wild type would indicate the number of T-DNA loci present in the mutant genome. Since kanR is a dominant effect, a ratio of 3:1 kanR : kanS seedlings in the F_2 generation would indicate that a single T-DNA locus was present in the genome, segregating in a normal Mendelian manner. Two independent T-DNA loci would result in a 15:1 ratio of kan R : kanS in the F_2 generation. I grew the seedlings on kanamycin plates with a 10x10 square grid superimposed on the petri dish with one seed per square, which facilitated the scoring of kanR and kanS plants. The segregation of the kanamycin resistance marker in the F_2 generation of the backcross to wild type is illustrated in table 3.6. The F_2 seed of *arc*6-1, *arc*7 and *arc*9 backcrossed to wild type segregated 3:1 for kanR : kanS indicating the presence of a single T-DNA locus in each mutant. The segregation of kanamycin resistance in the F_2 of the *arc*10 backcross was 15:1 kanR : kanS, suggesting the activity of two independently segregating T-DNA loci.

The 100% cosegregation of the T-DNA, revealed by kanamycin resistance, with the *arc* mutant phenotype in the F_3 progeny of the backcross to wild type would indicate that the *arc* mutant was co-located with the kanR marker; i.e. the *arc* mutant was tagged with a T-DNA. To assay for the cosegregation of the kanamycin resistance marker with the mutation, F_3 generation seed was taken from mutant F_2 plants of the backcross of mutant to wild type and sown on kanamycin plates. A result of 100% kanR in F_3 progeny of the mutant siblings would indicate that the mutant was tagged with a T-DNA. The results, summarised in table 3.6, demonstrate a segregation of kanR in the pooled numbers of mutant F_3 seedlings obtained from several mutant F_2 plants of *arc*6-1, *arc*7, *arc*9 and *arc*10. The evidence that the T-DNA may segregate away from the *arc* locus in these mutants suggests that none of the mutants contain a functional T-DNA which tags the mutant *arc* locus. The lack of any cosegregation of kanR with the mutation in *arc*10 suggests that neither of the two T-DNA loci are co-located with the *arc*10 mutant locus. Since *arc*6-2 and *arc*8 are both kanS it may be summarised that none of the *arc* mutants are tagged with a T-DNA which is detectable by the kanamycin resistance assay.

3.2.6 The analysis of the T-DNA inserts in arc *mutants*

The determination of the presence and orientation of the T-DNA inserts in the T-DNA mutagenised mutants was carried out by Southern blotting of genomic DNA extracted from mutant leaves. Figure 3.1 illustrates the composition of the 3850:1003 T-DNA construct used by Feldmann and co-workers for the seed infection mutagenesis (Feldmann and Marks, 1987, Errampalli, Patton, Castle *et al*, 1991).

The digestion of the mutant genomic DNA with *Hin*dIII provided an initial indication of the complexity of the various T-DNA loci when probed with pBR322, left border and right border probes. The autoradiographs obtained from these Southern blot hybridisations are illustrated in figure 3.18. The WS wild type control genomic DNA shows no reaction to either of the three T-DNA probes. *arc*6-1 shows a simple structure for the T-DNA; the mutant hybridises with the left border probe at a single band and with two bands for the pBR322 probe. *arc*6-1 DNA does not hybridise with the right border probe and exhibits an increased size of the 4.4kb pBR322 sequence from the expected 7.4kb restriction fragment recognised by the pBR322 probe to an 8kb fragment. These observations suggest a significant deletion of the right border sequences in *arc*6-1, including the *Hind*III site at the edge of the 4.4kb pBR322 sequence. The mutants *arc*7, *arc*9 and *arc*10 exhibit complicated patterns of bands when probed with left and right border probes, suggesting multiple inserts of some complexity. The sizes and intensity of the bands lit up by the pBR322 probe in *arc*7, *arc*9 and *arc*10 suggests that these mutants contain multiple intact pBR322 sequences of the predicted size. *arc*8 demonstrates no hybridisation to any of the T-DNA probes, suggesting the lack of a T-DNA in the *arc*8 mutant genome, consistent with the *arc*8 kanS phenotype.

A further investigation of the complexity and orientation of the T-DNA inserts was undertaken by the hybridisation of the pBR322 T-DNA probe to genomic DNA of the mutants cut with one of three restriction enzymes - *Hind*III, *Eco*RI and *Sal*I (figure 3.19). The predicted fragment sizes from these digests are indicated in figure 3.1.

FIGURE 3.18 Wild type and *arc* mutant genomic DNA probed for T-DNA

Autoradiograph of a single Southern blot of wild type and *arc* mutant genomic DNA of *Arabidopsis thaliana* ecotype WS hybridised to ^{32}P-labelled pBR322 *(a)*; Left Border*(b)*; Right border *(c)*. WS wild type, *arc*6-1, *arc*7, *arc*8, *arc*9 and *arc*10 DNA was cut with *Hin*dIII restriction enzyme. The location of size markers of λ DNA cut with *Eco*RI and *Hin*dIII are indicated at the left hand side of the autoradiograph. λ size markers are in kilobase pairs.

Figure 3.18

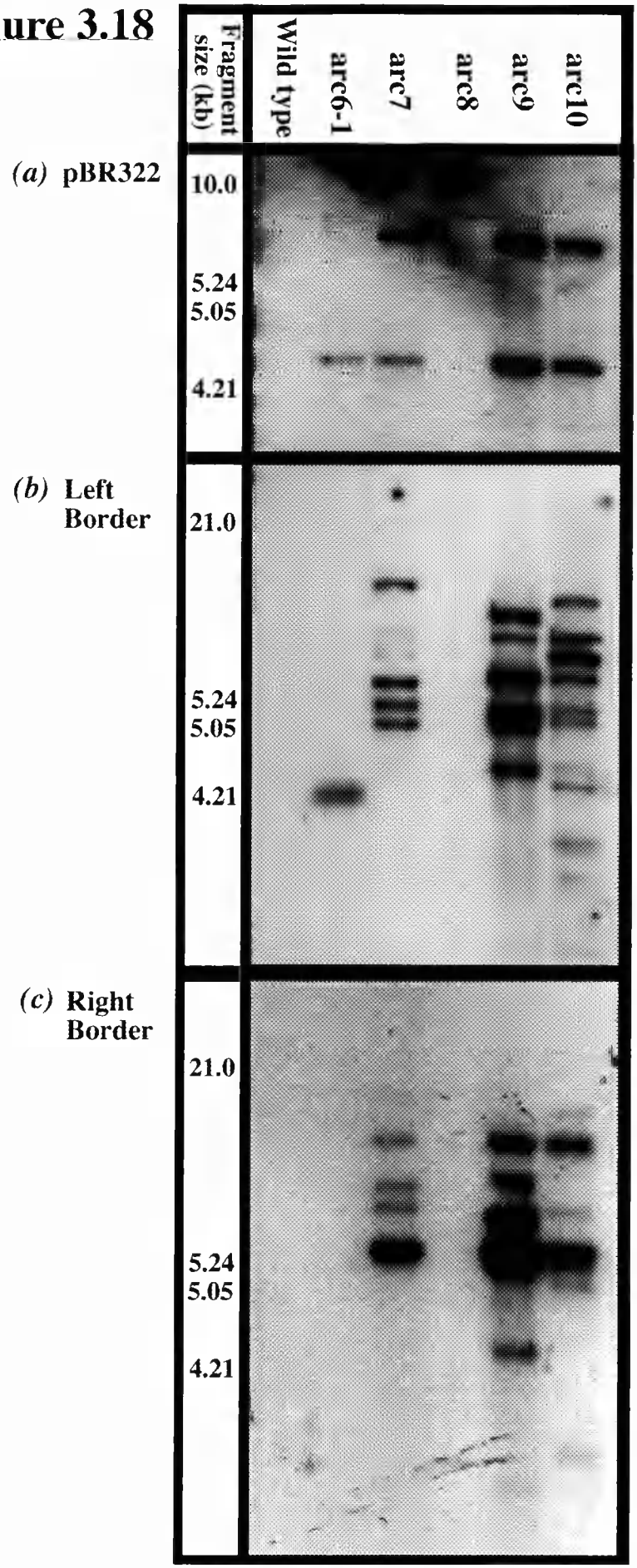
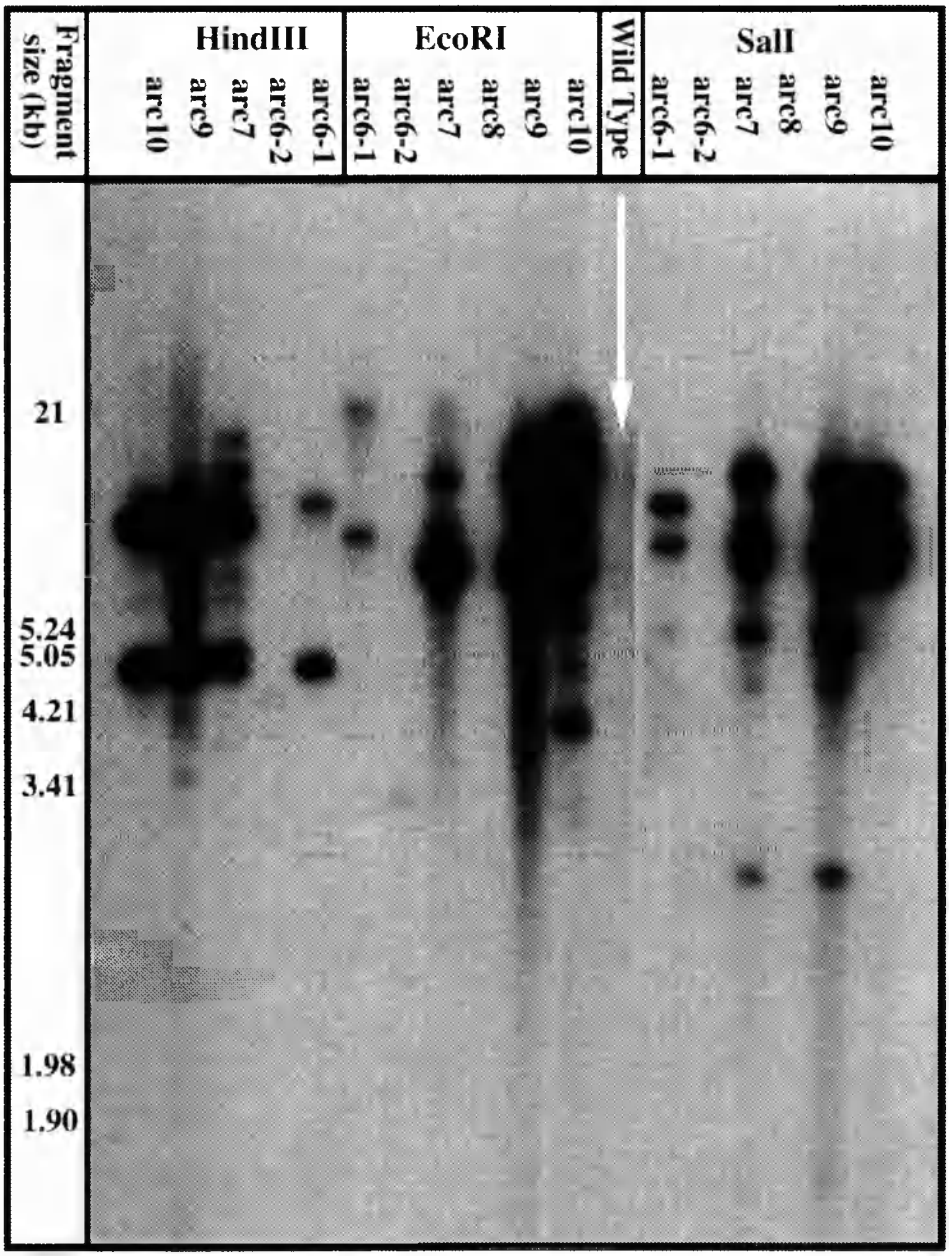

Fragment size (kb)
Wild type
arc6-1
arc7
arc8
arc9
arc10
(a) pBR322
10.0
5.24
5.05
4.21
(b) Left Border
21.0
5.24
5.05
4.21
(c) Right Border
21.0
5.24
5.05
4.21

FIGURE 3.19 Wild type and *arc* mutant genomic DNA digested by 3 enzymes and probed for T-DNA

Autoradiograph of Southern blot of wild type and *arc* mutant genomic DNA of *Arabidopsis thaliana* ecotype WS hybridised to ^{32}P -labelled pBR322. WS wild type DNA was cut with *Hin*dIII restriction enzyme. DNA from *arc*6-1, *arc*6-2, *arc*7, *arc*8, *arc*9 and *arc*10 mutants was cut with (*a*) *Eco*RI, (*b*) *Hin*dIII, (*c*) *Sal*I restriction enzymes. The locations of size markers of λ DNA cut with *Eco*RI and *Hin*dIII are indicated at the left hand side of the autoradiograph. λ size markers are in kilobase pairs.

Figure 3.19


Fragment size (kb)
HindIII
arc10
arc9
arc7
arc6-2
arc6-1
EcoRI
arc6-1
arc6-2
arc7
arc8
arc9
arc10
Wild Type
SalI
arc6-1
arc6-2
arc7
arc8
arc9
arc10
21
5.24
5.05
4.21
3.41
1.98
1.90

The comparison of the fragment sizes shown between the various mutants in the autoradiographs illustrated in figures 3.18 and 3.19 allow a prediction of the composition of the T-DNA loci in the T-DNA mutagenised mutants, summarised in figure 3.20.

***arc*6-1** is kanR and shows normal hybridisation to the left border and 3.8kb pBR322 fragments. The mutant does not hybridise to the right border and displays a large band for the 4.4kb pBR322 sequence. The 6.1kb band of the *Sal*I digest of *arc*6-1 probed with pBR322 suggests that the 4.4kb pBR322 is not significantly deleted. The 9kb band observed in the *Eco*RI digest indicates the absence of the *Eco*RI site. The *arc*6-1 mutant therefore is deleted at the right border losing an *Eco*RI site, but is otherwise complete.

***arc*6-2** is kanS and shows no hybridisation to LB or RB probes. The small fragment which hybridises to the pBR322 probe exhibits the same size with *Hind*III and *Sal*I, and a 3.1kb band with *Eco*RI. This suggests a severe truncation of the T-DNA, deleting the left border up to the *Hind*III and *Sal*I sites in the 1'NPTII gene, the 1'NPTII gene and a fraction of the 4.4kb pBR322 sequence with the right border.

***arc*7** exhibits no deletion of the left border or right border areas in a *Hind*III digest. The 14+kb and 6kb fragments of the *Sal*I digest indicate left border and right border inverted repeats respectively. The *Eco*RI fragment sizes indicate that the right border is external to the T-DNA.

***arc*8** is kanS and shows no hybridisation to the T-DNA probes.

***arc*9** shows similar characteristics to the T-DNA orientation in the *arc*7 mutant.

***arc*10** exhibits a complex structure of inverted repeats of both the left and right borders (*Sal*I 14 and 6 kb fragments). The lack of appropriate *Sal*I fragments of the correct size for the left border flanking sequences and large *Eco*RI fragments suggests that the right border of the *arc*10 T-DNA is external in both T-DNA loci.

3.2.7 The confirmation of the absence of silent T-DNA in arc6, arc7 and arc9

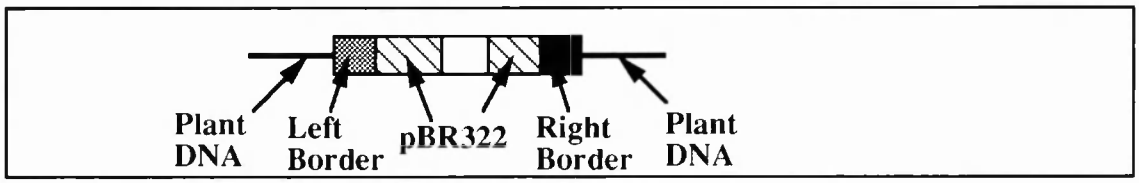
The reliability of the 1'NPTII gene for kanamycin resistance in *Arabidopsis* is not 100% (R. Schmidt, M. Bennett personal communication); an inactive 1'NPTII gene may therefore cause the T-DNA to be kanS. Such a '*silent*' T-DNA could potentially be the mutagen of an *arc* mutant but would be undetectable by the kanamycin resistance assay

FIGURE 3.20 Summary of T-DNA orientations in *arc* mutants

Diagrammatic representation of the predicted content and orientation of the T-DNA inserts in *arc* mutants of *Arabidopsis thaliana*, ecotype WS.
(*a*) 3850:1003 T-DNA with plant flanking sequences, Left border, Right border and pBR322 sequences indicated; (*b*) *arc*6-1; (*c*) *arc*6-2; (*d*) *arc*7; (*e*) *arc*8, (*f*) *arc*9; (*g*) *arc*10.

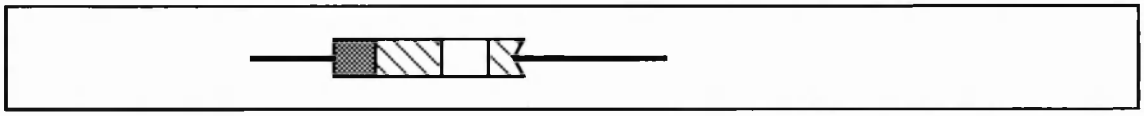
Figure 3.20

(*a*) **T-DNA - KEY**

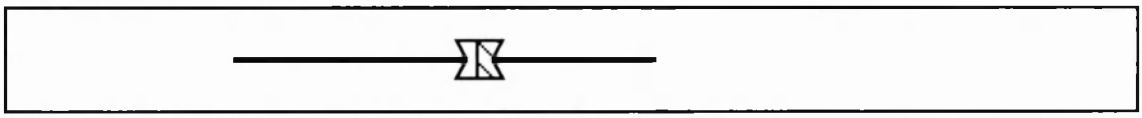


arc mutants

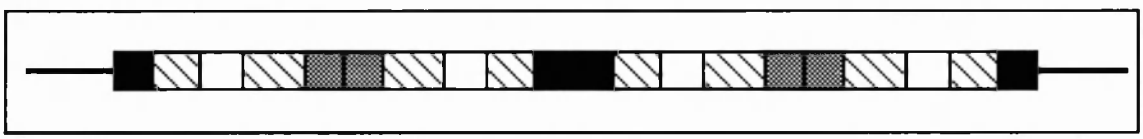
(*b*) ***arc*6-1**



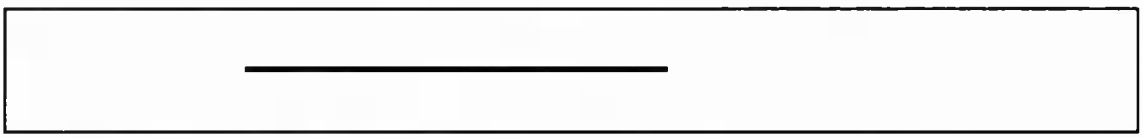
(*c*) ***arc*6-2**



(*d*) ***arc*7**

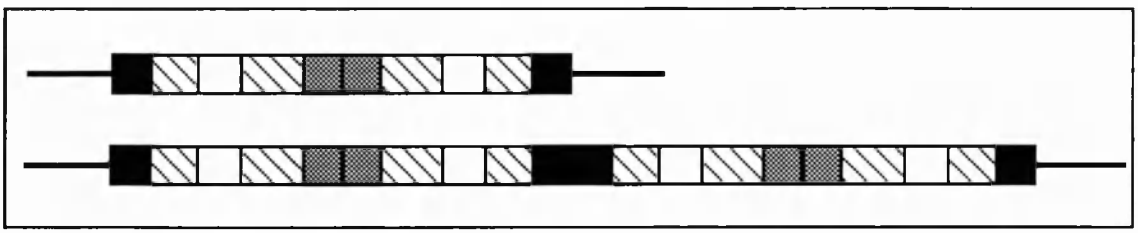


(*e*) ***arc*8**



(*f*) ***arc*9**

(*g*) ***arc*10**



(Castle, Errampalli, Atherton *et al*, 1993). The presence of silent T-DNAs in the *arc* mutant genome would be revealed by hybridisation of the T-DNA probes to the DNA of F_3 mutant plants which were also kanS. A further study of the apparently untagged mutants *arc*6-1, *arc*7 and *arc*9 was therefore undertaken.

Genomic DNA was prepared from bulked progeny seedlings obtained from mutant F_2 plants which segregated either homozygous kanR, heterozygous kanR/kanS or homozygous kanS in the F_3 generation. The presence of bands of kanS mutant DNA which hybridise to T-DNA probes on a Southern blot would reveal the presence of a silent T-DNA. Figure 3.21 illustrates the result of a hybridisation of pBR322 probe to *Hind*III digested genomic DNA of homozygous kanR, heterozygous kanR/kanS and homozygous kanS DNA for *arc*6, *arc*7 and *arc*9. No pBR322 hybridisation is noted in any of the kanS samples, indicating that there are no silent T-DNAs which may have caused the *arc*6-1, *arc*7 or *arc*9 mutations.

3.2.8 The analysis of the tagging of the arc*11 mutant with the* Ac *transposon*

The investigation of the tagged nature of the *arc*11 mutant was undertaken by the analysis of the cosegregation of the *Ac* element with the mutant phenotype and by the isolation of revertant individuals in the progeny of a mutant parent. The description of the tagging experiments for *arc*11 is detailed in chapter 6.

The *arc*11 mutant appears to be tagged with a functional *Ac* element, suggested by the existence of progeny of a single mutant parent displaying a wild type chloroplast phenotype indicative of somatic and germinal revertant individuals in the progeny .

3.2.9 Summary of the tagged status of the mutants

The tagged nature of the mutants *arc*6 to *arc*11 is summarised in table 3.7, including an indication of the potential of each mutant for isolation of the mutated gene locus. None of six T-DNA mutant alleles have proven to be tagged by a functional T-DNA insert. The cause of the mutations of these mutant alleles is not clear, although it is possible that *arc*6-2 is caused by a severely truncated pBR322 fragment. The other five T-DNA alleles may also be caused by insertion of fragments of the T-DNA undetected by the

FIGURE 3.21 Genomic DNA of kanR and kanS *arc* mutants probed for T-DNA

Autoradiograph of a Southern blot of genomic DNA from wild type, *arc* mutant and *arc* mutant backcrossed lines of *Arabidopsis thaliana* ecotype WS. *arc*6-1, *arc*7, WS wild type and *arc*9 DNA was cut with *HindIII* restriction enzyme and hybridised to ^{32}P -labelled pBR322 *(a)* and Left Border *(b)* DNA probes. Mutant genomic DNA was obtained from bulk harvests of parental *arc* mutant seedlings and F_3 seedlings from individual mutant F_2 progeny of a backcross of *arc* mutants to L.*er* wild type. F_3 seedling DNA was taken from progeny of F_2 mutant individuals which are either homozygous kanR/kanR, homozygous kanS/kanS or heterozygous kanR/kanS (tested in the F3 generation). The lack of hybridisation of the pBR322 probe to kanS/kanS DNA indicates the lack of a silent T-DNA insert in the individual. The locations of size markers of λ DNA cut with *Eco*RI and *Hin*dIII are indicated at the left hand side of the autoradiograph. λ size markers are in kilobase pairs.

Figure 3.21

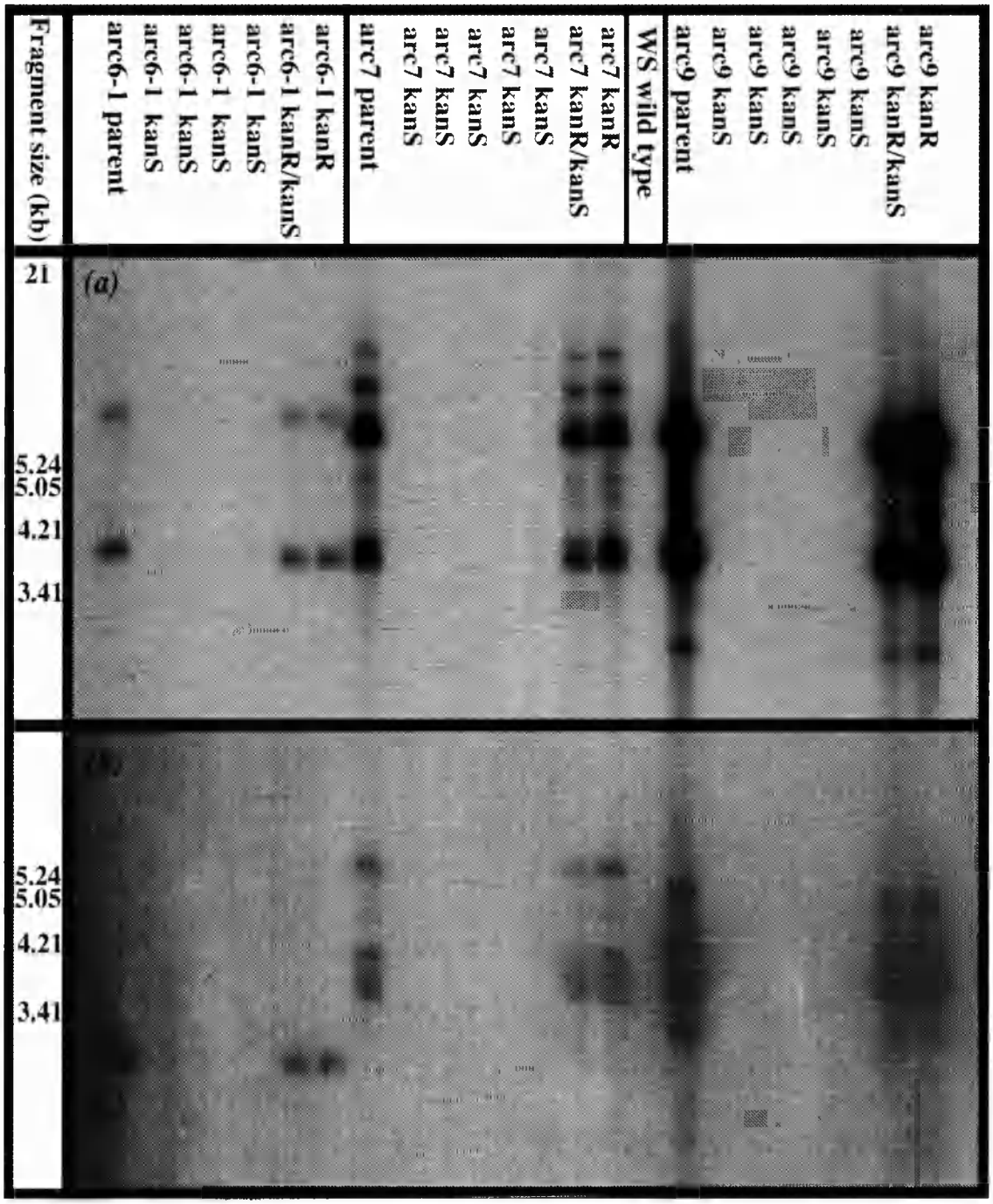

Fragment size (kb)
arc6-1 parent
arc6-1 kanS
arc6-1 kanR/kanS
arc6-1 kanR
arc7 parent
arc7 kanS
arc7 kanS
arc7 kanS
arc7 kanR/kanS
arc7 kanR
WS wild type
arc9 parent
arc9 kanS
arc9 kanS
arc9 kanS
arc9 kanR/kanS
arc9 kanR
21
5.24
5.05
4.21
3.41
(a)
(b)

Table 3.7

Summary of the tagged status of *arc* mutants

The tagged nature and character of inserted DNA mutagen of *arc* mutants of *Arabidopsis thaliana* ecotype Landsberg *erecta* and WS from transposon and T-DNA insertional mutagenised populations. Orientation and content of the T-DNA insertion loci determined by Southern blot hybridisation of left and right border T-DNA probes to *Hin*dIII digested genomic DNA and pBR322 hybridised to *Hin*dIII, *Eco*RI and *Sal*I digested DNA. The tagged status of the mutants was determined by cosegregation of the T-DNA kanamycin resistance marker (*arc*6 to *arc*10) or the *Ac* sequence (*arc*11) to the mutant loci and reversion of the mutant phenotype to wild type (*arc*11).

MUTANT ALLELE	POPULATION	DESCRIPTION OF INSERTION
***arc*6-1**	T-DNA N2646	Single insert. T-DNA truncated at right border
***arc*6-2**	T-DNA N2606	Single insert. pBR322 sequence , severely truncated
***arc*7**	T-DNA N2612	Single insert. T-DNA in sequence of inverted repeats
***arc*8**	T-DNA N2619	No T-DNA insert detectable
***arc*9**	T-DNA N2624	Single insert. T-DNA in a sequence of inverted and tandem repeats
***arc*10**	T-DNA N2629	Two T-DNA inserts. Several inverted and tandem repeats
***arc*11**	Transposon 02213-3 Line 122	Single copy of an active *Ac* element variation in chloroplast size. *arc*11 May revert to wild type phenotype ***arc*11 is TAGGED**

left border, right border and pBR322 probes, however the potential usefulness of such insertions for the isolation of the mutant genes is minimal. None of the mutant alleles isolated from the T-DNA-mutagenised populations is useful for the isolation of the mutant locus by plasmid rescue of the T-DNA. The *arc*11 mutant is tagged with a functional *Ac* element which can facilitate the isolation and characterisation of the *ARC*11 locus (discussed in chapter 6).

3.3 DISCUSSION

The screening of three populations of *Arabidopsis thaliana*, mutagenised with EMS chemical mutagen; the T-DNA of *Agrobacterium tumefaciens*; and the Δ*Nae*I *Ac* transposable element, has enabled the isolation of mutants of eleven independent nuclear loci. All of the mutants isolated are recessive mutant alleles of nuclear loci which are stable over several generations and which segregate in a normal Mendelian manner.

The mutants cover a broad range of mutant chloroplast phenotypes, as summarised in table 3.2. The relative values and demerits of each mutant for the further study of chloroplast division is also summarised in table 3.2. The *arc* mutants may be categorised into six classes dependent on the mutant chloroplast phenotypes:

(i) More, smaller chloroplasts than wild type (*arc*1 and *arc*7).

(ii) Variable chloroplast numbers and sizes (*arc*2, *arc*4 and *arc*11).

(iii) Two distinct sizes of chloroplast (*arc*9 and *arc*10).

(iv) Slightly fewer, larger chloroplasts than wild type (*arc*8).

(v) Significantly fewer, larger chloroplasts than wild type (*arc*3 and *arc*5).

(vi) Minimal numbers of extremely large chloroplasts (*arc*6).

The variety of *arc* mutant phenotypes is more diverse than one would have expected from a process as important as chloroplast accumulation, which is so closely related to the function of the mesophyll cell. However, the phenotypes display several similarities which demonstrate the toleration of the *Arabidopsis* cell for variations in its chloroplast complement.

The increase of chloroplast number with mesophyll cell expansion observed in the wild type is observed in all of the mutants which accumulate chloroplasts during mesophyll cell development. The mutants displaying a conserved chloroplast size such as *arc*1, *arc*7 and *arc*8 display a close relationship between chloroplast accumulation and cell expansion, while the mutants with more variable chloroplast sizes, *arc*2, *arc*9, *arc*10 and *arc*11 display a less conserved relationship. This is probably indicative of the greater degree of variation in the distribution of chloroplast sizes in the latter mutants. The mutants *arc*3, *arc*5 and *arc*6 which show no increase in chloroplast number do, however, display a significant degree of conservation of the original chloroplast number as the mesophyll cell expands. The variation in chloroplast numbers is also well compensated for by an inverse variation in chloroplast size so that as the mesophyll cell develops a constant proportion of the cell is covered by the chloroplast population. This maintenance of a constant chloroplast cover per cell is likely to account for the lack of a visibly deleterious effect of most *arc* mutants to the development of the whole plant. The constant chloroplast cover per cell, despite a wide variation in chloroplast number and size, clearly shows the degree of plasticity evident in the development of the *Arabidopsis* mesophyll cell.

The chloroplast number of the smallest, post-mitotic cells is noted to be approximately 14 in all the wild types and *arc* mutants except *arc*6. The complement of 14 plastids is almost certainly, therefore, the number of chloroplasts which arise from the proplastids partitioned into each of the post-meristematic cells. The constant number of 14 proplastids per cell between all of the *arc* mutants, except *arc*6, indicates that these mutants are mutations of *chloroplast* division and not *proplastid* division. The lack of a lesion in proplastid division in *arc* mutants with extremely diverse chloroplast numbers illustrates that chloroplast and proplastid division are two separate processes, affected by different genetic controls. The *arc*6 mutant chloroplast number never increases beyond 4, suggesting that the *arc*6 mutation affects proplastid accumulation as well as that of the chloroplast accumulation; this effect is further discussed in chapter 5.

The visualisation of CtDNA by the fluorescence of DNA-bound DAPI in PEG-embedded sections (3.2.2*(v)*) suggests that the CtDNA concentration is constant per cell despite variations in chloroplast number and size. This suggests that CtDNA replication

occurs relatively independent of chloroplast division so that the overall concentration of CtDNA per cell, and the ratio of CtDNA to NcDNA is constant between mutants and wild type despite extreme variations in chloroplast number. The preliminary data presented in 3.3.5 also suggest that there is no significant alteration to the ratio of CtDNA to NcDNA in the chloroplast complement as a whole per cell, although slight variations may be caused, as in *arc*1, *arc*7, and *arc*6. The CtDNA concentrations of the different *arc* mutants suggests that CtDNA replication is relatively independent of chloroplast division. It has been observed that CtDNA replication in wild type usually precedes chloroplast division. However, my results suggest that CtDNA replication does not itself initiate chloroplast division and is not significantly affected by a perturbation of chloroplast division in the mesophyll cell. These observations are of significant interest to the understanding of the replication and partitioning of CtDNA in the mesophyll cell, and require a more detailed analysis of the accumulation of CtDNA in the mutant chloroplasts before any conclusions may be drawn.

The wide diversity of the mutant chloroplast phenotypes in the eleven *arc* mutants compared to the relative lack of a detrimental mutant plant phenotype illustrates the extreme plasticity of the accumulation of the cell's chloroplast population. This plasticity suggests that chloroplast division may not be absolutely essential to the function of the mesophyll cell or the whole plant in *Arabidopsis*, since mesophyll cells of *arc* mutants may clearly function in the absence of any chloroplast division and questions the necessity for chloroplast division in *Arabidopsis*. The high degree of conservation of the wild type phenotype within an ecotype, however, suggests that there is an optimum for the size and number of *Arabidopsis* mesophyll cell chloroplasts for maximum efficiency of the *Arabidopsis* plant. There is presumably a disadvantage to the fitness of the cell caused by an altered chloroplast complement, because the wild type chloroplast phenotype of each ecotype is very conserved. The *arc* mutants do not exhibit a reduced fitness compared to wild type under controlled plant growth conditions. An investigation of the relative fitness of *arc* mutants compared to wild type in more natural conditions for *Arabidopsis* may reveal a reduction in fitness or another more extreme deleterious effect caused by the *arc* mutant chloroplast phenotype in particular environmental conditions.

Each of the *arc* mutants is of considerable value for the experimental study of mesophyll cell development in *Arabidopsis*. The mutant phenotypes of the *arc* alleles have demonstrated several characteristics of the development of the chloroplast complement, a major cytoplasmic compartment in the higher plant mesophyll cell. The importance of physical constraints such as cell and chloroplast size, observed by previous researchers, are also illustrated by the *arc* mutants and are discussed in chapter 4. The co-ordination of the nuclear and chloroplast genomes has also been highlighted by the *arc* mutants.

The screen of 13 000 seedlings of the T-DNA-mutagenised population represents approximately 30% of the original transformants in the population by a normal distribution; our screen yielded five independent *ARC* loci from this small percentage. The EMS screen performed by Pyke and Leech (1991) screened only 3000 seedlings, isolating at least five independent loci. This high incidence of *arc* mutants strongly suggests that the number of *ARC* loci in *Arabidopsis* is considerably higher than might have been predicted.

The high number of *ARC* loci in the *Arabidopsis* genome suggests that the genetic control of chloroplast division is a very complex process. Chloroplast replication would therefore appear to be a finely adapted process affected by several independent physical processes in the cell. A strong physical control apparently restricts the accumulation of the total chloroplast complement to a rigidly defined proportion of the mesophyll cell during cell expansion. This is shown by the tight restriction of this accumulation between a wide variety of genetic mutants which display very varied chloroplast phenotypes, and between the wild types of three different ecotypes. The conservation of the constant proportion of chloroplast cover per cell across such a wide genetic diversity indicates that it is controlled by a physical restraint. The results presented in chapter 4 also suggest strong physical controls to the initiation of chloroplast division. It is therefore likely that the mode of action of several of the *ARC* genes is coincidental to chloroplast division and accumulation rather than a direct control, such as the action of the plastid dividing ring. Furthermore, the high degree of integration of the chloroplast function with that of the cell (Kirk and Tilney-Bassett, 1978) would suggest that the replication of the plastid was also greatly influenced by the cell.

The several *arc* mutant phenotypes show similarities between general traits, but are individual and different between each other. Based on the indications of the various traits, speculations may be made as to the mutant effect and the function of the relevant *ARC* gene, as follows:

(i) arc6 The mode of action of the *arc*6 mutation is considered in more detail in chapter 5. The extremely low chloroplast number of *arc*6 suggests that the mutation is caused, at least in part, by a lesion in proplastid replication since the *arc*6 mutant does not have the base number of 14 chloroplasts observed in all other *arc* mutants. The mutant phenotype of *arc*6 proplastids in meristematic cells, discussed in chapter 5, supports this observation. Whether the *arc*6 mutation also affects chloroplast division in addition to proplastid division is not clear since the size and shape of the mutant proplastids when they develop into young chloroplasts may itself inhibit chloroplast division.

(ii) arc7 The *arc*7 mutant displays a chloroplast mutant phenotype of many, small plastids associated with a slight reduction in cell size. *arc*7 seedlings develop at a slightly slower rate than wild type and *arc*7 has a mutant plant phenotype of pale leaves in the young seedling which eventually attain wild-type colouration at *c*.28 days. The pale early plant phenotype may to be due to a retardation in the accumulation of photosynthetic pigments by the chloroplast which would retard photosynthetic capacity, resulting in *arc*7 lagging behind the wild type in developmental rate during early plant growth.

Several theories may explain the *arc*7, and also *arc*1, chloroplast mutant phenotypes. Firstly, the chloroplast division process may proceed at a faster rate than in wild type, allowing for increased numbers of divisions. Secondly, the period of cell development exhibiting optimum numbers of chloroplast division may be prolonged, possibly due to the retarded development of the *arc*7 seedling, allowing for more divisions to occur. A third suggestion is that chloroplast expansion is perturbed, either causing the *arc*7 chloroplast to remain at the optimum size for chloroplast division for an extended period, increasing the likelihood of chloroplast division occurring. The most likely explanation in view of the phenotype, however, is that the optimum size for the division of *arc*7 chloroplasts is reduced. The expansion of *arc*7 chloroplasts between divisions is

therefore restricted due to premature division at a smaller chloroplast size than the optimum noted in wild type.

The mean *arc*7 chloroplast size is approximately half the optimum size for chloroplast division in wild type (table 3.1, Chapter 4) suggesting that very few, if any, *arc*7 chloroplasts attain the optimum size required for normal division. The increase in chloroplast division in *arc*7 indicates that chloroplast division occurs more frequently in *arc*7 mesophyll cells than in wild type, the optimum size for chloroplast division must therefore be reduced in the *arc*7 mutant. Since the *arc*7 cells maintain the constant proportion of the cell covered by the chloroplast complement observed in wild type, an increased rate of chloroplast division would not result in increased chloroplast number per cell, compared to wild type, unless the chloroplasts are also able to divide without expanding to the optimum size for division. A reduction in the expansion of chloroplasts between sequential divisions is therefore the most likely explanation for the *arc*7 chloroplast phenotype, since the other theories for increased chloroplast number suggest that the *arc*7 chloroplasts attain the wild type optimum size for chloroplast division which clearly does not occur.

(iii) arc8 The chloroplast number of *arc8* mesophyll cells is half that of wild type. The chloroplast phenotype of *arc*8 suggests that there is an anomaly in chloroplast division which impedes chloroplast division without abolishing the process. The lack of significant numbers of chloroplast division profiles in *arc*8 mesophyll cells suggests that the initiation of the chloroplast division process is retarded rather than the completion. Chloroplast divisions therefore occur less frequently in *arc*8 than wild type, but once initiated, proceed to completion at the normal rate. However, all chloroplasts must undergo the same number of divisions since the size of *arc*8 chloroplasts is relatively uniform. The cause of such a retardation in chloroplast division is unknown. The moderate chloroplast and whole plant mutant phenotype of *arc*8 may suggest that a less radical alteration to *arc*8 cell development may induce the *arc*8 effect, rather than an inhibition in the process of chloroplast division.

(iv) arc9 and arc10 The *arc9* and *arc10* mutants are the only *arc* mutants with phenotypes which display significantly different sub-populations of chloroplasts of two different sizes within a single cell. The *arc2* and *arc*11 phenotypes also display highly variable chloroplast numbers and sizes (section 6.3.1, Pyke and Leech 1992), but these phenotypes do not have chloroplasts of two distinct size categories, as seen in *arc*9 and *arc*10. The most probable explanation for the existence of two populations of chloroplast sizes may be the differential rate of chloroplast divisions within the same cell. Possingham (1980) suggests that all chloroplasts divide in the wild type cell, however the existence of a few very large chloroplasts in the *arc*9 and *arc*10 cells strongly suggests that these particular chloroplasts have not divided at all and were prevented from doing so in the early cycles of division. The smaller chloroplasts of *arc*9 and *arc*10 cells are generally of a uniform size slightly larger than wild type, and are likely, therefore, to have undergone division at a reduced rate possibly due to a similar effect to that of *arc*8.

(v) arc11 The *arc*11 and the *arc*2 mutants display chloroplast numbers and sizes which are highly variable in the mesophyll cell. The range of chloroplast size appears to be a random distribution between small chloroplasts and chloroplasts three fold larger than wild type. The *arc*11 mutant is more extreme in this variable chloroplast mutant phenotype than *arc*2. The apparently random variation in chloroplast mutant phenotype between cells (figure 3.9, table 3.1) is also noted in the chloroplast number per mesophyll cell so that the relationship between chloroplast number and chloroplast size is less tightly correlated than is observed in the majority of *arc* mutants.

The variation in chloroplast size and number in *arc*11 and *arc*2 suggests a differential frequency of chloroplast division between the chloroplasts of an individual mesophyll cell so that a spectrum of chloroplast numbers and sizes is the result. This theory is discussed further in chapter 4.

Predictions of the mutant effects of the various *arc* mutants are presently limited to speculation, until the ARC gene products have been isolated and characterised from cloned and sequenced *ARC* genes. The isolation of *ARC* loci by gene tagging of *arc* mutants has

not been as straightforward as was originally anticipated. This is because the six novel mutant phenotypes from the T-DNA mutagenised population are of recessive alleles and represent five independent nuclear loci. The *arc*11 mutant, isolated from the transposon-mutagenised population, however, is the only *arc* mutant which is tagged with a DNA insertion which is usable for the isolation of the wild type *ARC*11 gene.

The alleles *arc*6-2 and *arc*8 are not kanamycin resistant, suggesting the lack of a functional T-DNA. The *arc*6-1, *arc*7, *arc*9 have one NptII-functional T-DNA present in the genome; *arc*10 contains two independent T-DNA loci. Segregation data of the NptII kanamycin resistance marker of the T-DNA with the mutant phenotypes indicates that neither the *arc*6-1, *arc*7, *arc*9 nor *arc*10 mutant alleles are tagged with a functional T-DNA. Southern blot analysis suggests that the T-DNA loci in *arc*7, *arc*9 and *arc*10 are complex structures consisting of inverted and tandem repeats of T-DNA sequences. The T-DNA of the *arc*6-1 allele is a single insert, truncated at the right border. The *arc*6-2 allele contains only a small, severely truncated fragment of pBR322 sequence. The *arc*8 allele does not contain a recognisable T-DNA sequence at all. The *arc*6-1, *arc*7 and *arc*9 alleles have been analysed for the presence of silent T-DNA inserts which do not convey resistance to kanamycin. No silent T-DNA loci were observed in the three alleles.

The lack of a tagged mutant allele in the *arc* mutants isolated from the T-DNA population is likely to be due primarily to bad fortune, since only six individual mutants were isolated. Castle, Errampalli, Atherton *et al* (1993) observed that approximately 65% of the 178 mutants, which they isolated from the Feldmann population, were *not* tagged with a T-DNA insert. The lack of a large collection of mutants in the *arc* mutant screen therefore significantly reduces the potential for isolating a tagged mutant. The absence of a significant mutant whole plant phenotype in the majority of *arc* mutants requires the labour-intensive analysis of mesophyll cells under the microscope, inhibiting the use of a mass screen for whole plant mutant phenotypes.

The complex T-DNA structures observed in the *arc*6-1, *arc*6-2, *arc*7, *arc*9 and *arc*10 mutant alleles is not an unusual characteristic of mutants from the Feldmann T-DNA mutagenised population (Castle, Errampalli, Atherton *et al*, 1993; Martineau, Voelker and Sanders, 1994). The presence of inverted and tandem repeats of the T-DNA is not

surprising since the integration of the *Agrobacterium* T-DNA is often characterised by duplications of the T-DNA (Martineau, Voelker and Sanders, 1994). The cause of the mutant phenotypes in the untagged *arc* alleles is not known. The small fragment of pBR322 sequence observed in *arc*6-2 is possibly the cause of the *arc*6-2 mutation. An analysis of the cosegregation of the pBR322 fragment with the *arc*6-2 mutant phenotype would verify this suggestion, but the presence of such a truncated fragment of the T-DNA is unlikely to be sufficient for the plasmid rescue of the *arc*6-2 allele as it does not contain the bacterial kanamycin resistance gene (NPT) required for the selection of successfully transformed host cells in plasmid rescue (Castle, Errampalli, Atherton, *et al*, 1993). The cause of the other *arc* mutants is less clear but may be due to several effects. For example, the untagged *Lfy* mutation (Weigel, Alvarez, Smyth, Yanofsky and Meyerowitz, 1992) has been shown to be caused by a single base mutation. The analysis of embryonic mutants isolated from the Feldmann populations (Castle, Errampalli, Atherton *et al*, 1993) has suggested that some mutations may have been caused by the insertion of *Agrobacterium* DNA sequences into the plant genome. Alternatively, the insertion of truncated fragments of the T-DNA not detectable by the left border, right border and pBR322 probes may have induced the mutations. Castle, Errampalli, Atherton *et al* have observed that approximately 20% of the embryonic mutants isolated from the Feldmann population have undergone chromosomal translocations, possibly due to inserted T-DNAs, which are of considerable size, causing inefficient chromosome pairing (Lee, 1988). Chromosomal rearrangement at the mutant locus has not been noted in mapping studies of *arc*6 (5.3.2), which contains a single copy of the T-DNA, however this may possibly be the cause of the mutations in *arc*7, *arc*9 or *arc*10 which appear to have larger T-DNA inserts. The detailed analysis of the sequence of the *arc* alleles after isolation of the genes by other strategies than T-DNA tagging will reveal the cause of the untagged mutations.

The isolation of the untagged mutant loci may be approached by one of two strategies, either *(i)* the screening of mutant populations for tagged alleles or *(ii)* the initiation of a map-based cloning strategy.

(i) The analysis of insertional mutagenised populations of *Arabidopsis* for a tagged *arc* allele would facilitate the isolation of the *ARC* gene. Such populations include the remainder of the Feldmann population yet to be screened, as well as other populations such as the vacuum-infiltrated T-DNA mutagenised populations of Sangwan and co-workers (Sangwan, Velu, Cobanov, *et al*, 1993) or the *Ac* and *Ac/Ds* mutagenised populations of Dean and co-workers (Dean, Sjodin, Lawson, Lister, Scofield and Jones (1990); Bancroft, Bhatt, Sjodin, Scofield, Jones and Dean, 1992; Dean, Sjodin, Page, Jones and Lister, 1992; Bancroft, Jones and Dean, 1993). Further screening for tagged mutants will require a sub-cellular screen since the majority of *arc* mutants have no whole plant mutant phenotype. The exception is the wrinkled leaf phenotype of *arc*6 which aids future screening strategies by removing the requirement to analyse each seedling microscopically for the *arc*6 mutation. The screening of mutagenised populations for alleles of *arc* mutants other than *arc*6 will be less efficient, requiring the intensive screening of several seedlings microscopically by eye.

(ii) The isolation of *arc* mutant loci by map-based cloning is a more time-consuming method for gene isolation than the use of gene tagging and the isolation of *ARC* genes by this approach would therefore be limited to only a few loci. The genetic mapping of *arc*6 has already been initiated and is described in chapter 5. The completion of a map-based cloning experiment *via* chromosome walking is a long-term prospect; however, the mapping of the various mutants to regions of the *Arabidopsis* chromosomes may enable the more rapid isolation of the *ARC* genes by the use of mutagenesis by localised transposition of mapped *Ds* elements (Long, Martin, Sundberg *et al,* 1993) or by the localisation of the mutant locus to one of the many regions of the *Arabidopsis* genome incorporated into a contiguous array of YAC or cosmid clones (Gibson and Somerville, 1992).

Future work

The tagged *arc*11 allele is presently being characterised and cloned; the initial stages of the cloning procedure are discussed in chapter 6. The map-based cloning of *arc*6 has also been initiated. The mapping of other *arc* alleles such as *arc*1, *arc*3 and *arc*5 may

provide an *arc* locus whose map location is ideal for cloning of the gene. The examination of mutant populations for tagged alleles of *arc*6 is clearly a priority.

The lack of tagged alleles other than *arc*11, however should not obscure the fact that there are now eleven characterised *arc* loci in *Arabidopsis* which may provide significant insight into the control and procedure of the chloroplast division process. The analysis of the ultrastructure of the *arc* mutants is being undertaken by E.J Robertson in our laboratory (some of whose results are presented in this thesis), and has revealed several characteristics of the *arc* mutant physiology which have proven invaluable in the study of the mutant phenotypes. The detailed analysis of the development of the individual mutants will also provide significant insight into the control of the process of chloroplast division in *Arabidopsis*. The co-ordination of the accumulation of the total chloroplast complement and its limitation by the cell is also a matter which deserves analysis. Some of the physical controls to chloroplast division are discussed in the following chapter.

An analysis of the replication of CtDNA in the mutants should also be initiated. The investigation of the apparent independence of the CtDNA replication to chloroplast division observed in the *arc* mutants is of great interest to the study of the co-ordination of the nuclear and cytoplasmic genomes during development in *Arabidopsis*.

The genetic interaction of the *arc* mutants has been studied in *arc*1, *arc*3 and *arc*5 (Pyke and Leech, 1994); the study of the interaction of the other *arc* alleles will enable the action of the *ARC* genes to be accurately located in the chloroplast division cycle. The analysis of homozygous double mutants of *arc* mutants with *arc*1 or *arc*7 will provide some indication of the severity of the mutant effect on the chloroplast and provide insights into the mode of action of the mutants which suppress chloroplast division.

3.4 SUMMARY

A screen of seedlings from T-DNA and transposon mutagenised populations of *Arabidopsis thaliana* was undertaken to isolate individuals mutant for chloroplast number and size in the mesophyll cells of the first leaf. The tagging of mutants by the insertion of T-DNA or transposon DNA fragment of known sequence was anticipated to facilitate the isolation of *ARC* genes. Seven individual recessive mutants representing six independent nuclear *ARC* loci were isolated and have been characterised for chloroplast number, chloroplast size and CtDNA content. The mutants represent phenotypes in which chloroplast division is either enhanced, reduced or completely suppressed and bear similar phenotypic traits to established *arc* mutants.

Six of the *arc* mutants, representing five independent *ARC* loci, were isolated from the T-DNA mutagenised population and analysed for cosegregation of the T-DNA with the mutant locus but were shown to be untagged with a functional T-DNA. One mutant, *arc*11, isolated from the transposon mutagenised population was demonstrated to be tagged with a functional *Ac* element.

CHAPTER 4

An Analysis of the Physical Control of Chloroplast Division in the *arc*5 Mutant and Landsberg *erecta* Wild Type

4.1 INTRODUCTION

The study of chloroplast division in *Arabidopsis* has been aided by the use of *arc* mutants of chloroplast division. A null baseline of a lack of chloroplast division provided by some *arc* mutants has shown the effect of chloroplast replication on the normal function of the cell and the nature of the control of chloroplast accumulation in mesophyll cells.

The ***arc*5** mutant is ideal for the analysis of the control of chloroplast division, since the *arc*5 mutant chloroplasts do not increase in number during cell development, indicating that chloroplast division does not occur in *arc*5. Almost all *arc*5 chloroplasts display a degree of central constriction, usually indicative of a dividing chloroplast (Leech, Thomson and Platt-Aloia, 1981) suggesting that the chloroplasts of *arc*5 have initiated division, but not completed the process. The *arc*1/*arc*5 double mutant (Pyke and Leech, 1994) which is homozygous for both the *arc*5 and the *arc*1 mutations expresses the *arc*5 mutant phenotype of reduced division in an *arc*1 background where division is increased. The *arc*1/*arc*5 double mutant chloroplast number is intermediate between *arc*5 and wild type and shows that *arc*5 does not completely inhibit division when placed in a non-wild type background. The intermediate double mutant phenotype is therefore of considerable use to the study of the *arc*5 mutant effect.

The *arc*5 mutant chloroplasts do not complete division but rather increase in size until the cellular maximum for total chloroplast area is reached. The accumulation of the photosynthetic complement in *arc*5 is therefore clearly not dependent on the cumulative divisions of chloroplasts, yet a normal total chloroplast area is attained in this mutant (figure 3.11). The study of chloroplast accumulation in *arc*5 compared to wild type was anticipated to provide an insight into the nature of the control of the accumulation of the chloroplast complement.

The physical control of the accumulation of the chloroplast complement is the central aim of the work presented in this chapter. Two physical parameters are investigated for their effect on chloroplast division and accumulation; these are chloroplast size and mesophyll cell size. Both of these physical factors have been suggested previously to have an effect on chloroplast accumulation in wheat (Ellis and Leech, 1985; Pyke and Leech,

1987; reviewed in chapter 1). The use of *arc*5 and the *arc*1/*arc*5 double mutant facilitates the study of the roles of cell size and chloroplast size in chloroplast division in both *Arabidopsis* wild type and in a mutant where the initiation of chloroplast division may proceed but its completion is inhibited.

The analysis of the accumulation of the numbers of dividing chloroplasts in expanding cells of Ler wild type and *arc*5 was undertaken to indicate the effect of cell size on the numbers of chloroplasts and the proportion of the total chloroplast number which are in division as the cell expands. A comparison of the sizes of dividing and non-dividing chloroplasts in wild type and *arc*5 was also undertaken to indicate any maximum, minimum or optimal chloroplast sizes at which division is initiated.

4.2 RESULTS

4.2.1 The mutant phenotype of arc5

The *arc*5 mutant mesophyll cell phenotype was studied in order to compare the development of the *arc*5 chloroplast population with that of wild type. A typical mesophyll cell of the *arc*5 mutant is illustrated in figure 4.1*(c)* showing a mutant phenotype with a small number of large chloroplasts, the majority of which exhibit a constriction around the equator of the plastid. The *arc*5 mesophyll cells contain approximately 14 chloroplasts, compared to the Ler wild type mean of 121 chloroplasts, and chloroplast number does not increase during mesophyll cell expansion (Pyke and Leech, 1994; figure 3.7*e*). Thus it is likely that chloroplast division is inhibited during mesophyll cell development in the *arc*5 mutant. Proplastid replication does not appear to be inhibited in *arc*5, since the smallest *arc*5 mesophyll cells contain approximately 14 chloroplasts, which is almost certainly the number of proplastids apportioned into each young *Arabidopsis* mesophyll cell (3.3.1). The *arc*5 mesophyll cell chloroplasts are approximately six fold larger than Landsberg *erecta* wild type chloroplasts in fully expanded mesophyll cells (Pyke and Leech, 1994, figure 3.9*e*). The increase in chloroplast size in *arc*5 and the reduction in chloroplast number compared to wild type compensate, thus maintaining a total chloroplast cover for the *arc*5 mesophyll cell which is not significantly different from wild type (figure 3.11*e*).

Almost all *arc*5 chloroplasts are observed to exhibit a constriction at the equator at right angles to the major axis of the chloroplast. The accumulation of the number of chloroplasts which show this constricted phenotype during cell expansion is discussed in 4.2.5. The constriction shown by the *arc*5 chloroplasts is similar in appearance to the central constriction indicative of dividing plastids in other species such as wheat (Leech, Thomson and Platt-Aloia, 1981), spinach (*Spinacea oleracea*) (Possingham and Lawrence, 1983) and oats (*Avena sativa*) (Oross and Possingham, 1989). Typical isolated *arc*5 and Landsberg *erecta* chloroplasts are illustrated in figure 4.2. The central constriction of chloroplasts has been used as an indication of division in the subsequent analysis of dividing chloroplasts in *Arabidopsis*.

Figure 4.1

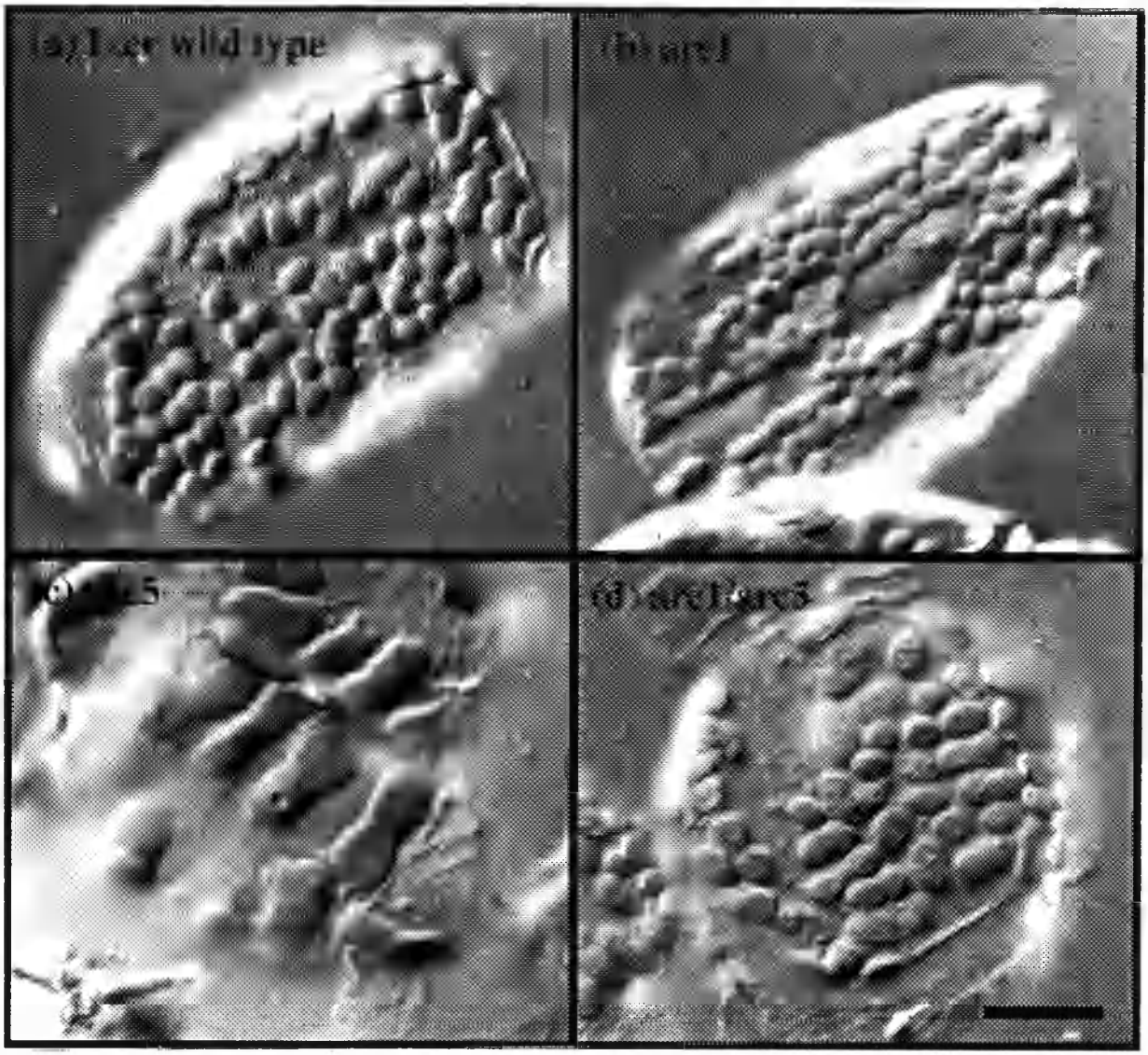


FIGURE 4.1 **Ler wild type, *arc*1, *arc*5 and *arc*1/*arc*5 double mutant mesophyll cells**

Photomicrographs of isolated mesophyll cells from fully expanded first leaves of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype Landsberg *erecta* viewed with Nomarski differential contrast optics. (*a*) Landsberg *erecta* wild type; (*b*) *arc*1; (*c*) *arc*5 (*d*) *arc*1/*arc*5 double mutant. Bar = 25μm.

Figure 4.2

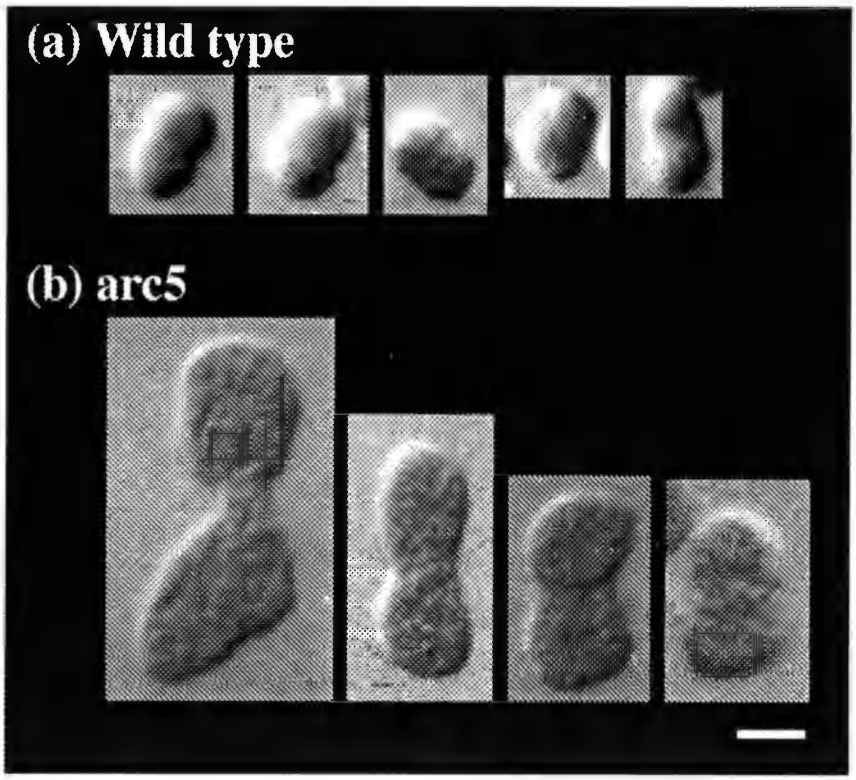


FIGURE 4.2 **Ler wild type and *arc*5 isolated chloroplasts**

Photomicrograph of isolated mesophyll cell chloroplasts from broken protoplasts of fully expanded first leaves of wild type and *arc*5 mutant of *Arabidopsis thaliana*, ecotype Landsberg *erecta* viewed with Nomarski differential contrast optics. (*a*) Landsberg *erecta* wild type; (*b*) *arc*5. Bar = 10μm.

4.2.2 The ultrastructure of constricted arc5 *chloroplasts*

At the same time as I was investigating *arc*5, Dr E. Robertson in our lab performed ultrastructural studies on the *arc*5 mutant chloroplasts using tissue which I had grown and embedded. When viewed under the electron microscope, I observed the ultrastructure of several young (7 day) and old (27 day) *arc*5 mesophyll cell chloroplasts. The electron micrographs of two *arc*5 chloroplasts shown in figure 4.3 are representative of chloroplasts from 7 day and 27 day old *arc*5 tissue.

The constricted *arc*5 chloroplast of the 7 day old first leaf mesophyll cell (illustrated in figure 4.3*(a)*) displays a comparable morphology to the dividing chloroplast in other species (Leech, Thomson and Platt-Aloia, 1981; Modrusan and Wrischer, 1990). The chloroplast is frequently constricted into an hourglass or dumb-bell shape by an isthmus which varies between being only moderately constricted to the extremely constricted morphology shown by the plastid in figure 4,3*(a)*. The central constriction is usually approximately central along the major axis of the plastid. The internal thylakoid membranes are distributed in approximately equal proportions between each half of the dividing 7 day old *arc*5 chloroplast and are compressed where the isthmus has constricted. A ring of opaque material was often observed across the narrow isthmus, and appears to be of a similar nature to the **plastid dividing ring** observed in previous studies (Leech, Thomson and Platt-Aloia, 1981; Oross and Possingham, 1989; reviewed in chapter 1). Observations of dividing chloroplasts in ultramicrographs have shown that one or more mitochondria are often present at the constricting isthmus of chloroplasts in division (Modrusan and Wrischer, 1990). A single mitochondrion was also frequently observed at the isthmus of the 7 day old dividing *arc*5 chloroplast, an example is shown in figure 4.3*(a)*, and in wild type dividing chloroplasts.

In comparison, the ultrastructure of the larger *arc*5 chloroplast from 27 day old leaf tissue shown in figure 4.3*(b)* is very different from the 7 day old tissue. The 27 day old *arc*5 chloroplasts are very large and often irregular in shape. The constricted appearance of the large chloroplast, characteristic of *arc*5, may be observed when the plastid is viewed longitudinally, as in figure 4.1. When the large *arc*5 chloroplast is viewed in transverse

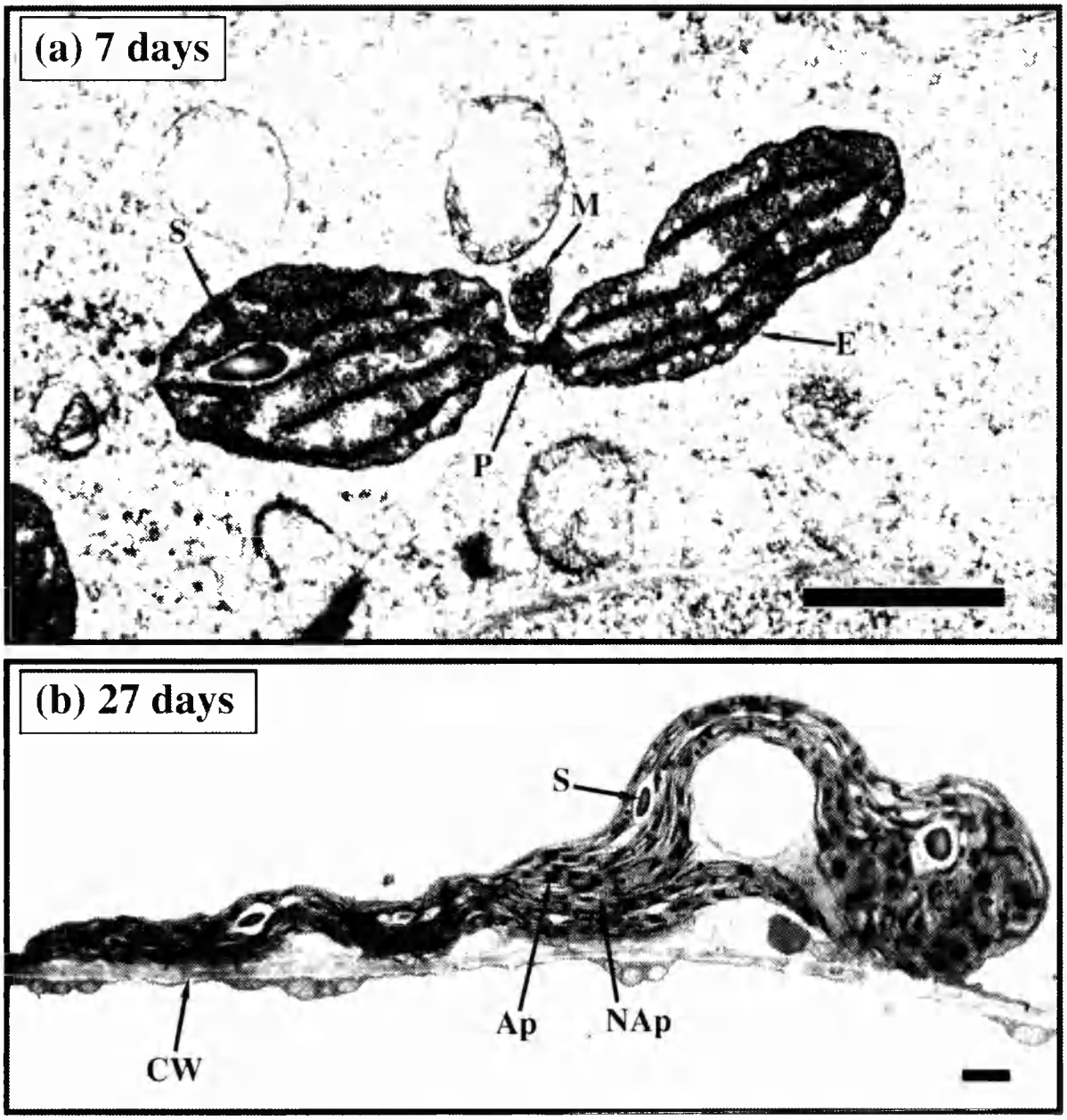
FIGURE 4.3 Ultrastructure of *arc*5 chloroplasts

Electronmicrograph of mesophyll cell chloroplasts of young and fully expanded first leaves of the *arc*5 mutant of *Arabidopsis thaliana*, ecotype Landsberg *erecta*. (*a*) 7 day old tissue; (*b*) 27 day old tissue. **C** = Chloroplast; **Ap** = Appressed membrane; **NAp** = Non-appressed membrane; **P** = Plastid dividing ring; **M** = Mitochondrion; **CW** = Cell Wall; **S** = Starch grain. Bar = 1μm.

Photographs by Dr E. Robertson.

Reproduced from Robertson, Rutherford, Pyke and Leech, in preparation.

Figure 4.3



section, the thin cross sectional area of the chloroplast reduces the extremity of the constriction so that the constricted phenotype of the plastid is not always clearly visible. The isthmus of the constricted *arc*5 chloroplast is not as tightly constricted in the older plastids as in the 7 day old plastids. The wider isthmus in the larger *arc*5 chloroplasts shows that the plastid dividing ring can relax from the tightly constricted form which was observed in the young *arc*5 chloroplasts. The internal structure of the large *arc*5 chloroplast is very contorted compared to the younger *arc*5 plastids. The thylakoid membrane is more prolific in the older *arc*5 chloroplasts than the 7 day old chloroplasts, and is often observed to be twisted or folded within the larger plastid. The twisting of the thylakoid membrane is shown in figure 4.3*(b)*, where the grana to the left hand side of the large strach grain in this plastid may be seen as stacks of membranes, whereas those in the right hand side of the starch grain are viewed from the top of the granal stack and seen as disks. The twisting of the thylakoid membrane by up to 90° is also observed in the dividing chloroplast in wheat (Leech, Thomson and Platt-Aloia, 1981). The overall shape of the 27 day old *arc*5 chloroplast is irregular and the surface of the chloroplast envelope undulates to a high degree. This irregular chloroplast shape is common in the larger more mature *arc*5 chloroplasts.

These observations on the early and later stages of the development of the *arc*5 chloroplast allow me to speculate that in early development the smaller *arc*5 chloroplasts begin to divide and almost complete a plastid division cycle since a centralised constriction and the plastid dividing ring are both present. However it is clear that in the *arc*5 mutant the chloroplasts do not complete chloroplast division, since in *arc*5 mesophyll cells chloroplast number does not increase during mesophyll cell expansion: in addition subsequent expansion is correlated with the widening of the constricted isthmus. In later development the thylakoid membranes of the large *arc*5 chloroplast may become contorted and twisted within the two ends of the dumb-bell shaped plastid, presumably due to differential expansion of the two parts of the constricted *arc*5 chloroplast. Thus the *arc*5 mutant chloroplast phenotype may reflect the effect of chloroplast expansion on plastids which do not complete the division process.

4.2.3 The phenotype of the arc*1*/arc*5 double mutant*

The *arc*5 mutant does not increase in chloroplast number during cell expansion, indicating that chloroplast division does not normally occur in this mutant. An intermediate phenotype between the normal chloroplast division of wild type and the inhibited chloroplast division of *arc*5 was required to effectively interpret the disparity between the two extreme phenotypes. The *arc*1/*arc*5 double mutant, constructed by Pyke and Leech (1994) (illustrated in figure 4.1*(d)*)was therefore included in this study since the double mutant chloroplast phenotype is intermediate between *arc*5 and wild type but is still homozygous for the *arc*5 mutation. The *arc*1/*arc*5 double mutant is homozygous for both the *arc*1 and *arc*5 mutant recessive alleles and the chloroplasts show the manifestations of the combined effect of both mutant genes. The *arc*1/*arc*5 double mutant mesophyll chloroplasts are more numerous than in the *arc*5 mutant (the *arc*1/*arc*5 double mutant mean chloroplast number is 49 per mesophyll cell, compared to the Ler mean of 121 and the *arc*5 mean of 14 chloroplasts per cell (Pyke and Leech, 1994)). These double mutant chloroplasts are smaller than *arc*5 chloroplasts and are only approximately twice the size of Ler chloroplasts in fully expanded mesophyll cells, compared to *arc*5 chloroplasts which are 6 fold larger than wild type. The *arc*1/*arc*5 double mutant also typically contains high numbers of constricted chloroplasts, *c*.50% of the total chloroplast population, discussed in 4.2.5.

The increase in chloroplast number in the *arc*1/*arc*5 double mutant clearly shows that the *arc*5 mutation is *not* a null effect which would completely inhibit all chloroplast division under its genetic influence, but rather an effect which retards chloroplast division to such an extent that in a non-*arc*1 background, chloroplast division does not normally occur. The analysis of the size of constricted chloroplasts in the *arc*1/*arc*5 double mutant supports this conclusion, since it suggests that the chloroplast division process is prolonged over a wider range of chloroplast sizes in the double mutant compared to wild type (4.3.5).

4.2.4 The effect of chloroplast size on chloroplast division in wild type, arc*5 and the* arc*1*/arc*5 double mutant mesophyll cells*

A relationship between chloroplast size and chloroplast division was discussed by Ellis and Leech (1985) who suggested that there is an optimum plastid size required for chloroplast division to occur. The large size of the *arc*5 chloroplasts which have not divided suggests that there may also be a maximum chloroplast size beyond which division is impossible. Since the appearence of the chloroplasts in the *arc*1/*arc*5 double mutant shows that the *arc*5 mutation does not completely inhibit chloroplast division; the lack of any division in *arc*5 chloroplasts may be the result of their increased size. The investigation of whether the chloroplasts of *Arabidopsis* have a minimum, maximum and optimum size for plastid division was undertaken by comparing the sizes of dividing and non-dividing chloroplasts in Landsberg *erecta* wild type, in *arc*5 and in the *arc*1/*arc*5 double mutant.

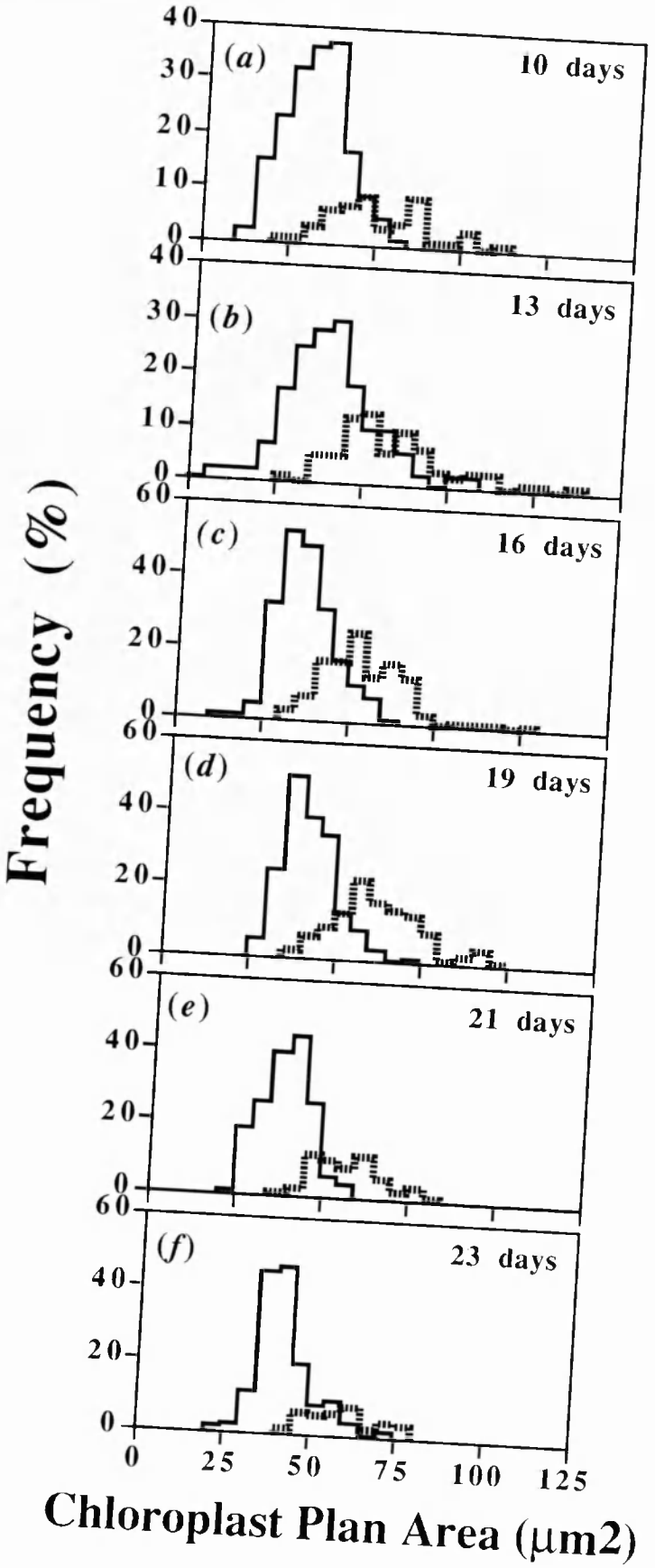
A sequence of chloroplast plan area distributions measured from mesophyll cells of Landsberg *erecta* wild type leaves of increasing age is shown in figure 4.4. The plan area distributions of constricted and non-constricted plastids are included in each sample. The mean plan area of non-dividing wild type plastids is noted as approximately 40μm^2, while the mean plan area of dividing chloroplasts is 60μm^2. The size of non-dividing wild type chloroplasts reaches a peak at 50μm^2. This 50μm^2 chloroplast size coincides with the plan area of the peak in the frequency distribution of the dividing chloroplasts. This co-ordination in the sudden reduction in frequency of non-dividing chloroplasts with the increase in frequency of dividing chloroplasts at 50μm^2 plan area suggests that chloroplast division is initiated optimally at a plastid size of 50μm^2 in *Arabidopsis* Ler wild type.

The existence of dividing chloroplasts smaller than the optimal 50μm^2 indicates that chloroplast division may occur, at a reduced frequency, in smaller plastids. The smallest chloroplasts exhibiting constriction in the younger sample ages are 20μm^2 increasing to 30μm^2 in older samples and are at least 10μm^2 larger than the smallest non-constricted chloroplasts, suggesting a minimum chloroplast size of approximately 25-30μm^2 for the initiation of chloroplast division.

FIGURE 4.4 Dividing and non-dividing chloroplast sizes in Ler wild type

Frequency distributions of chloroplast plan area for dividing (dashed line) and non-dividing (solid line) mesophyll cell chloroplasts from first leaves of wild type *Arabidopsis thaliana*, ecotype Landsberg *erecta*. Samples taken at ages (*a*) 10, (*b*) 13, (*c*) 16, (*d*) 19 and (*e*) 23 days.

Figure 4.4


(a)
10 days
(b)
13 days
(c)
16 days
(d)
19 days
(e)
21 days
(f)
23 days
Frequency (%)
Chloroplast Plan Area (µm2)
0
25
50
75
100
125

The largest non-dividing chloroplasts in wild type are approximately 65μm^2, although plastids are also observed which are 75μm^2 in plan area. Since no non-dividing chloroplasts are noted beyond this range of sizes it may be assumed that the large dividing chloroplasts observed (which may reach up to 115μm^2) are dividing plastids in the later stages of division rather than those in which division has been recently initiated. These observations therefore would suggest that in Landsberg *erecta* the maximum size at which chloroplast division may be initiated is approximately 65μm^2. The division process, once initiated, may however proceed in chloroplasts up to a maximum size of *c.*115μm^2.

The plan areas of the dividing chloroplasts are larger than the majority of the non-dividing chloroplasts. This discrepency in size shows that in *Arabidopsis*, chloroplast expansion continues throughout chloroplast division so that chloroplasts complete division at a larger size than when division was initiated. The *arc*5 chloroplast phenotype of several large chloroplasts which show division characteristics also suggests that chloroplast expansion may proceed, even once division is initiated.

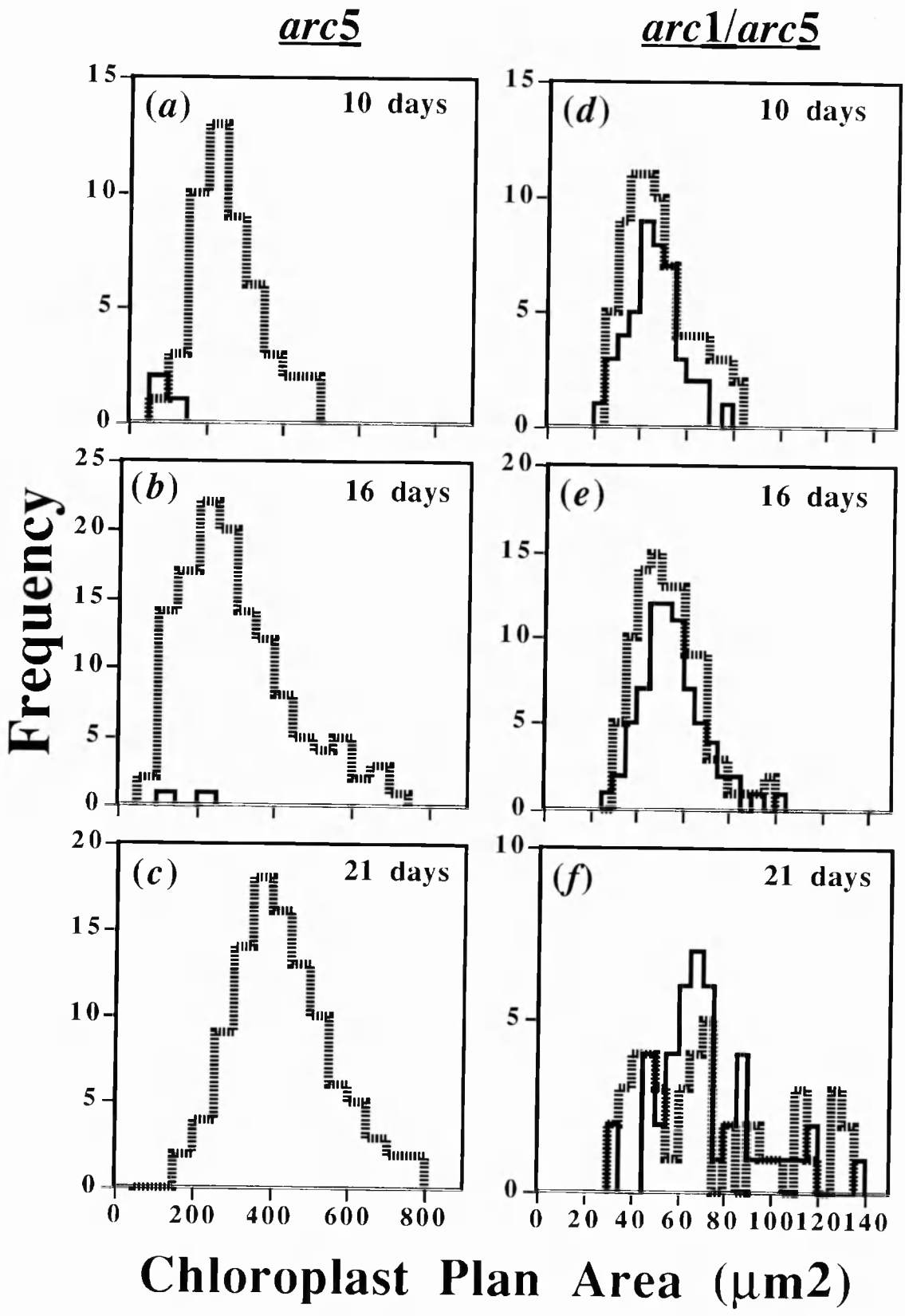
The smallest constricted *arc*5 chloroplasts observed are 50μm^2 (figure 4.5*(a) and (b)*) supporting the suggestion that this is the optimum size for the initiation of chloroplast division in Landsberg *erecta Arabidopsis* mesophyll cells. Since *arc*5 chloroplasts do not complete division and rather expand whilst still displaying a division profile, the majority of dividing *arc*5 chloroplasts are larger than 50μm^2.

The dividing chloroplasts of the *arc*1/*arc*5 double mutant cover a wide range of sizes (figure 4.5*(d)* - *(e)*). The majority of chloroplast divisions in the *arc*1/*arc*5 double mutant are initiated at less than 50μm^2 chloroplast plan area, but the chloroplasts may maintain a division profile in 16 day old tissue for up to 100μm^2, similar to the maximum size for division noted in wild type. This would suggest that in the *arc*1/*arc*5 double mutant, chloroplast division may be initiated at any plan area within a range of sizes which is broader than both wild type and *arc*5. This broader range of sizes in which the *arc*1/*arc*5 double mutant chloroplast may divide may explain why chloroplast division can proceed in *arc*1/*arc*5 even in the presence of the *arc*5 mutation. The increase in chloroplast number

FIGURE 4.5 Dividing and non-dividing chloroplast sizes in *arc*5 and the *arc*1/*arc*5 double mutant

Frequency distributions of chloroplast plan area for dividing (dashed line) and non-dividing (solid line) mesophyll cell chloroplasts from first leaves the *arc*5 mutant and *arc*1/*arc*5 double mutant of *Arabidopsis thaliana*, ecotype Landsberg *erecta*. (*a*) *arc*5 10 days, (*b*) *arc*5 16 days, (*c*) *arc*5 21 days, (*d*) *arc*1/*arc*5 10 days, (*e*) *arc*1/*arc*5 16 days, (*f*) *arc*1/*arc*5 21 days.

Figure 4.5


arc5
arc1/arc5
(a) 10 days
(b) 16 days
(c) 21 days
(d) 10 days
(e) 16 days
(f) 21 days
Frequency
Chloroplast Plan Area (μm2)

in *arc*1/*arc*5 shows that the *arc*5 mutation clearly does not completely inhibit division but rather retards it so that in the *arc*5 mutant, chloroplast division cannot occur before the chloroplast expands beyond the maximum size for division. A premature initiation of division in *arc*1/*arc*5 probably extends the period in which division may proceed, so that even the *arc*5 mutant effect does not inhibit division.

The results of the above analyses would indicate that in *Arabidopsis* ecotype Landsberg *erecta* chloroplast division is initiated in chloroplasts between 25μm^2 and 65μm^2 and may proceed to completion in wild type chloroplasts which are already in division up to a maximum plan area of 115μm^2. Chloroplast division, however, is initiated optimally at a plan area of 50μm^2 in Landsberg *erecta* wild type. This size is considerably larger than the 5μm^2 plan area suggested as the optimum chloroplast size for division in wheat (Ellis and Leech, 1985).

4.2.5 The effect of leaf and mesophyll cell expansion on chloroplast division in wild type, arc*5 and the* arc*1*/arc*5 double mutant*

The expansion of the mesophyll cell influences chloroplast division in a number of species (Leech and Pyke, 1988; Possingham, Hashimoto and Oross, 1989). The close association of increasing chloroplast number with increasing mesophyll cell size in wheat (Pyke and Leech, 1987) and *Arabidopsis* (Pyke and Leech, 1991) suggests that cell size limits chloroplast accumulation during development. Cell size is probably the most important restrictive factor of the mesophyll cell on the control of chloroplast division in *Arabidopsis*.

Mesophyll cell expansion has also been suggested to be a factor which can initiate the replication of the chloroplast complement as well as restrict the accumulation of the chloroplast population as division proceeds (Pyke and Leech, 1987). The *arc*5 mutant chloroplast phenotype indicates a lesion in chloroplast division; however the lack of chloroplast division is compensated for by chloroplast expansion. The total chloroplast cover in *arc*5 is therefore similar to wild type. Since cell size is not different between *arc*5

and wild type, despite radical differences in chlorplast number, it is likely that cell size is a major factor in the control of chloroplast accumulation in *arc*5. The comparison of the accumulation of dividing chloroplasts relative to cell expansion in *arc*5 compared to wild type was anticipated to indicate the effects of cell size on chloroplast accumulation in *Arabidopsis*.

(*a*) Landsberg *erecta* wild type

The relationship between the number of dividing chloroplasts per mesophyll cell and leaf age in first leaf samples of Landsberg *erecta* wild type of ages ranging from 10 to 23 days old was first determined. The results of this analysis (figure 4.6) show that the proportion of chloroplasts in division increases to a maximum mean value of 28% in 13 day old tissue. This maximal level reduces in older tissue to a mean of 5% in the oldest tissue samples. These results would indicate an initial stimulation of chloroplast division in the young tissue.

The number of chloroplasts in division relative to mesophyll cell expansion in Landsberg *erecta* wild type is shown in figure 4.7*(a)*. There are increases in the numbers of constricted chloroplasts during early mesophyll cell expansion, with a peak of approximately 10 dividing chloroplasts per cell in mesophyll cells of 1600 μm^2 plan area. The number of chloroplasts exhibiting constriction is then reduced to a constant number of 7 per cell as mesophyll cell expansion proceeds.

The proportion of the total number of chloroplasts in division in expanding Ler mesophyll cells, shown in figure 4.7(*b*), indicates that there is a maximum of 26% of total chloroplasts which are undergoing constriction at a mesophyll cell plan area of approximately 1500μm^2. The proportion of chloroplasts in division declines during subsequent mesophyll cell expansion and only 5% are in division in mesophyll cells greater than 5000μm^2 plan area. Possingham (1980) and Leech and Pyke (1988) suggest that in many plants, *all* chloroplasts of the mesophyll cell divide during the normal development of the chloroplast complement. However it is clear that in *Arabidopsis,* not all chloroplasts in a cell divide at once, even when chloroplast division is first initiated.

Figure 4.6

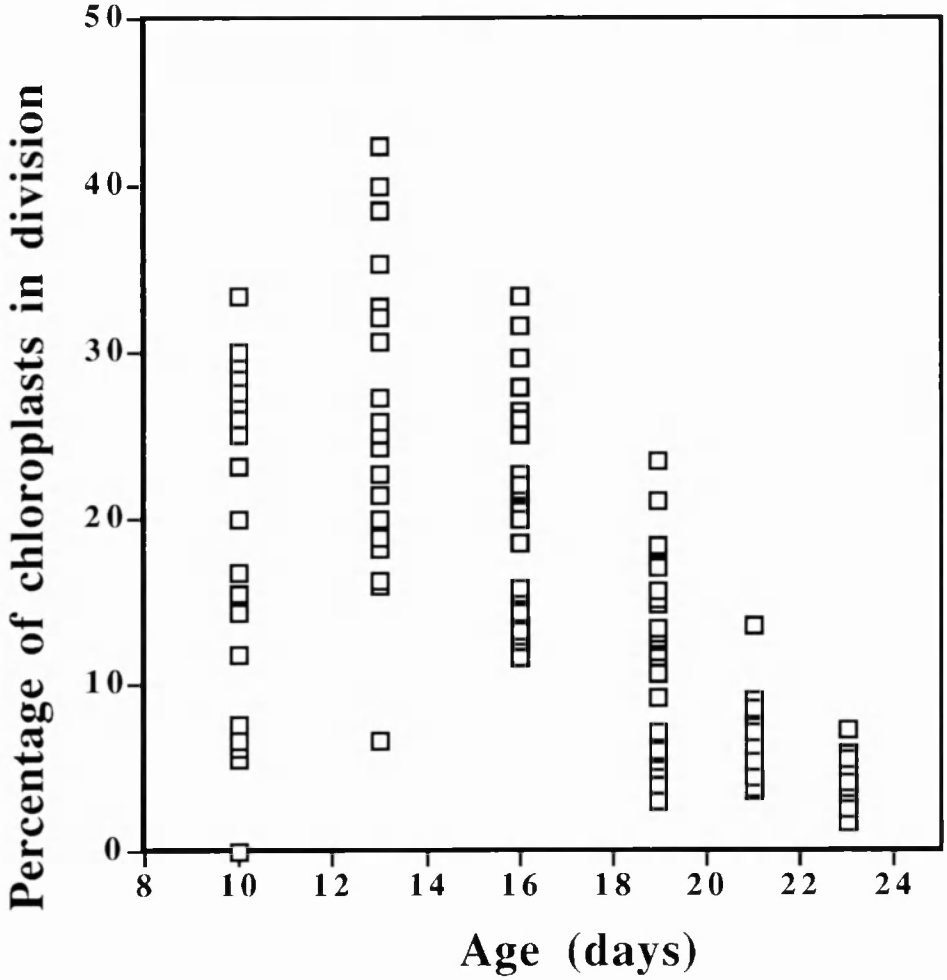


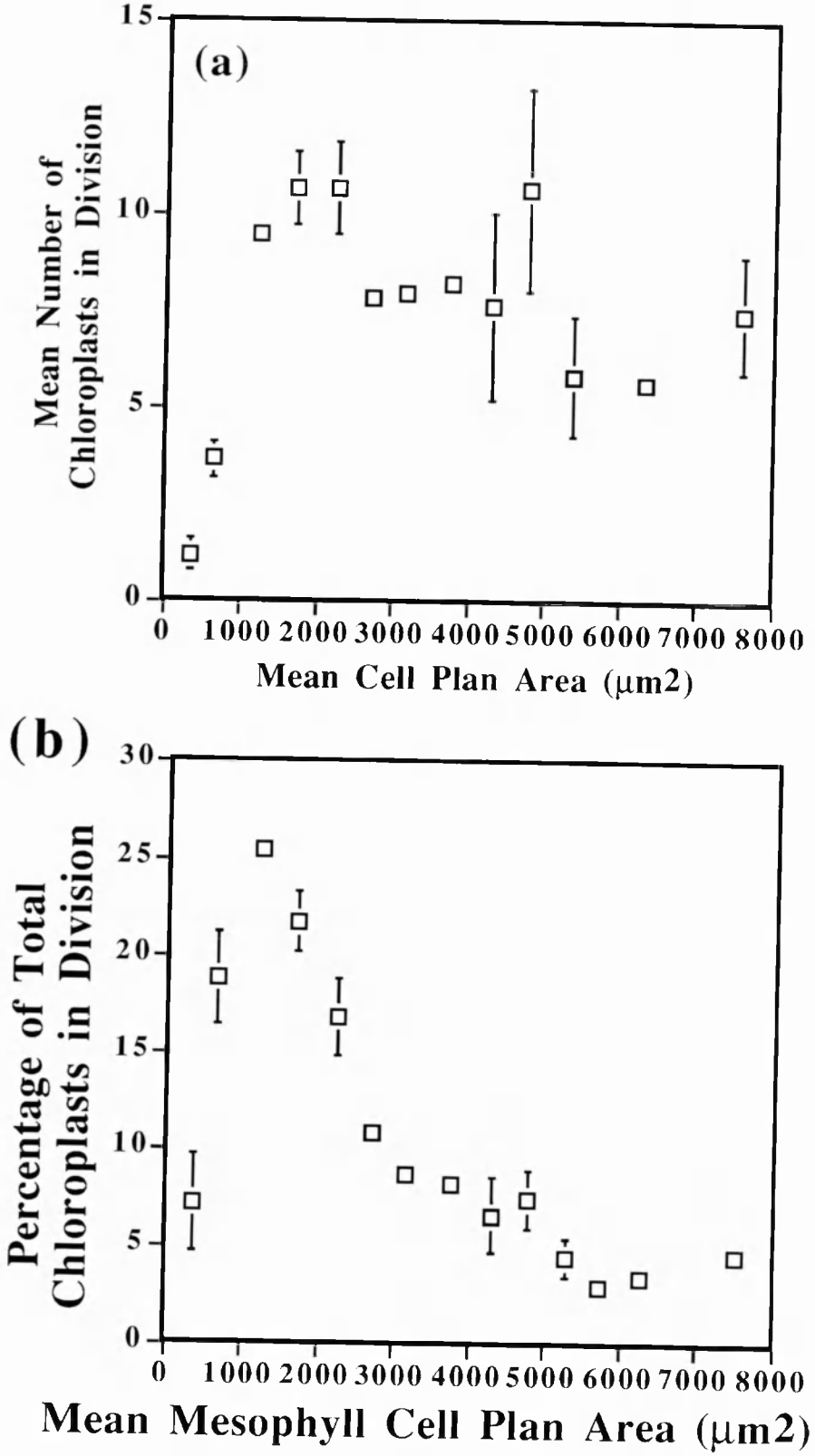
FIGURE 4.6 **Leaf age and chloroplast division in Ler wild type**

The relationship between the proportion of total number of chloroplasts in division per mesophyll cell for leaf sample ages 10 to 23 days of first leaves of wild type *Arabidopsis thaliana*, ecotype Landsberg *erecta*. Each data point represents the measurement from one cell.

FIGURE 4.7 Mesophyll cell size and chloroplast division in Ler wild type

The relationship between (*a*) the number of chloroplasts in division per mesophyll cell and (*b*) the proportion of the total number of chloroplasts in division per mesophyll cell and mesophyll cell plan area for wild type *Arabidopsis thaliana*, ecotype Landsberg *erecta*. Each data point represents the mean of all cell measurements per 500 μm^2 range of cell plan area, standard error bars are illustrated but may be smaller than the data point symbol.

Figure 4.7


(a)
Mean Number of Chloroplasts in Division
15
10
5
0
0 1000 2000 3000 4000 5000 6000 7000 8000
Mean Cell Plan Area (μm2)
(b)
Percentage of Total Chloroplasts in Division
30
25
20
15
10
5
0
0 1000 2000 3000 4000 5000 6000 7000 8000
Mean Mesophyll Cell Plan Area (μm2)

(*b*) *arc*5 and the *arc*1/*arc*5 double mutant

In contrast to wild type, almost 100% of the chloroplasts in each mesophyll cell of the *arc*5 mutant phenotype exhibit a constriction. Figure 4.8 shows the relationship between the proportion of the total chloroplast number which are in division and mesophyll cell plan area in *arc*5 and in *arc*1/*arc*5 plants. The proportion of visibly constricted chloroplasts in *arc*5 increases from 10% to almost 100% of chloroplasts per mesophyll cell, reaching the maximal proportion at a mesophyll cell plan area of 2500μm^2. Clearly in the *arc*5 mutant all the chloroplasts begin chloroplast division, even if division is not completed. This observation is consistent with the theory that in normal plants all chloroplasts per mesophyll cell can, and may, undergo division at some point during the development of the chloroplast complement.

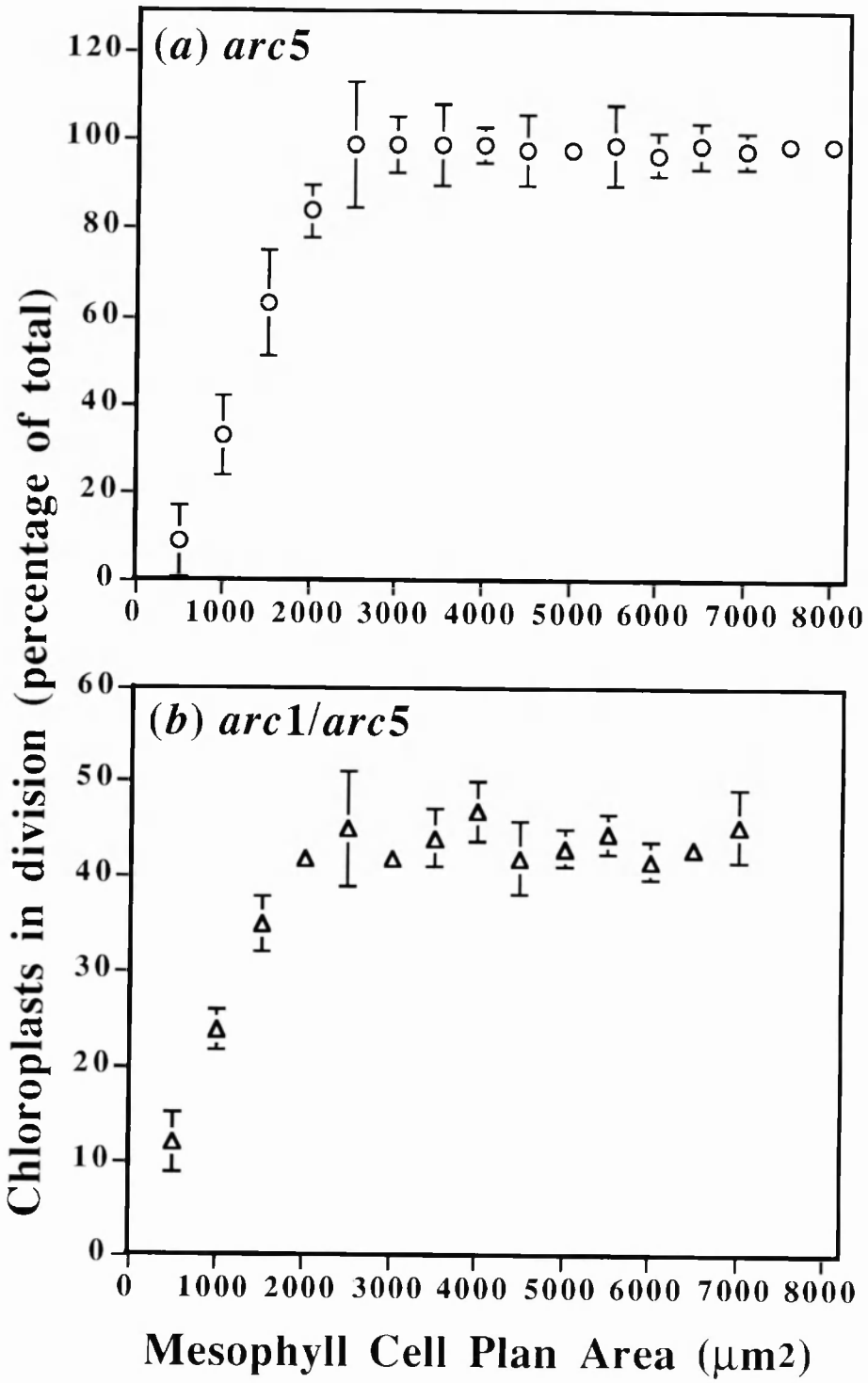
In the mesophyll cells of the *arc*1/*arc*5 double mutant the proportion of chloroplasts in division increases from 10% to approximately 45% in cells of c. 2600μm^2 plan area. The proportion 45% of chloroplasts which are in division does not decrease as is observed in wild type cells (figure 4.7 *(b)*), suggesting that chloroplast division proceeds at a slower rate in the *arc*1/*arc*5 double mutant than in wild type. The reduction in division rate would cause an increase in the proportion of chloroplasts that are observed as dumb-bells at any given time during development.

These comparisons of the proportions of the chloroplast complement which are in division in wild type and *arc*5 illustrates three important points. Firstly, low cell size is associated with rapidly increased proportions of dividing chloroplasts per cell, although even in these cells there is never a majority of chloroplasts in division at one time. Secondly, all chloroplasts in the normal *Arabidopsis* mesophyll cell probably undergo division during cell development. Lastly, chloroplast divisions are not synchronous with one another so that only a small proportion of the total chloroplast complement are in division at any particular phase of cellular development.

FIGURE 4.8 Mesophyll cell size and chloroplast division in *arc*5 and the *arc*1/*arc*5 double mutant

The relationship between (*a, c*) the number of chloroplasts in division per mesophyll cell and (*b, d*) the proportion of the total number of chloroplasts in division per mesophyll cell and mesophyll cell plan area for *arc*5 mutant and *arc*1/*arc*5 double mutant of *Arabidopsis thaliana*, ecotype Landsberg *erecta*. Each data point represents the mean of all cell measurements per 500μm range of cell plan area, standard error bars are illustrated but may be smaller than the data point symbol.

Figure 4.8


(a) arc5
120
100
80
60
40
20
0
0 1000 2000 3000 4000 5000 6000 7000 8000
(b) arc1/arc5
60
50
40
30
20
10
0
0 1000 2000 3000 4000 5000 6000 7000 8000
Chloroplasts in division (percentage of total)
Mesophyll Cell Plan Area (μm2)

4.3 DISCUSSION

The *arc*5 mutant phenotype of chloroplasts which are arrested in division is one of the most useful *arc* mutant phenotypes yet isolated in *Arabidopsis*, and its study has considerably extended our understanding of the genetic and physical control of chloroplast division. In the *arc*5 mutant the constriction of dividing chloroplasts occurs, but the daughter plastids do not separate so that division rarely, if ever, is completed in the chloroplasts of *arc*5 mesophyll cells. The comparison of the accumulation of the chloroplast population in *arc*5 and in Landsberg *erecta* wild type indicates that several factors are involved in the control of the process of chloroplast division in *Arabidopsis*.

Low chloroplast numbers and the large chloroplast size in the mesophyll cells of the *arc*5 mutant may be the result of one of two effects:-

(i) The completion of division in *arc*5 chloroplasts may be retarded sufficiently that the chloroplast expands beyond the size at which division may again occur. Therefore the chloroplasts can expand continuously in the absence of successful division to a size beyond which chloroplast division is not possible under normal conditions. A plant expressing the *arc*5 mutation in the *arc*1 background, such as the *arc*1/*arc*5 double mutant, is clearly capable of chloroplast division at a limited rate, suggesting that the *arc*5 mutation does not completely inhibit chloroplast division, but rather retards it. There is almost certainly a maximum size for chloroplast division, otherwise the chloroplasts of the *arc*5 mutant would be expected to be able to complete at least one division during mesophyll cell expansion. An analysis of the sizes of wild type dividing chloroplasts suggests that there is a maximum size beyond which chloroplast division may not initiate, and a further maximum beyond which a previously initiated chloroplast division may not be completed. The expansion of the incompletely divided *arc*5 chloroplast beyond this maximum size is likely to be the major factor which halts the accumulation of chloroplast number in the mutant.

(ii) The alternative means by which the *arc*5 mutation could be effective would be as a result of the increased rate of expansion of the *arc*5 plastid so that the maximum size for chloroplast division was exceeded before division could be completed. This

explanation would support an optimum and a maximum size for chloroplast division and a continuous background of chloroplast expansion. However, such an effect would require very rapid expansion of the chloroplast, since the division process itself is a rapid event (Leech, Thomson and Platt-Aloia (1981) suggest it takes 20 minutes in wheat). Furthermore, the *arc*5 chloroplast has been shown to be constricted to a considerable degree, as shown by figure 4.3 *(b)*, suggesting that all the events up to the very final stages of division may proceed, even in small *arc*5 chloroplasts. It is unlikely that this is the explanation for the *arc*5 mutant chloroplast phenotype

The lack of tightly constricted isthmi in the large *arc*5 chloroplasts, compared to the extreme constriction of the young *arc*5 dividing chloroplast isthmus, suggests that as the *arc*5 chloroplast expands, the plastid dividing ring at the isthmus also slips open so that the central constriction becoming wider. This suggests that the constriction of the plastid dividing ring is not an irreversible process during chloroplast division in *arc*5. The *ARC* 5 gene may therefore be necessary for the efficient function of the plastid dividing ring so that without the *ARC*5 gene effect, constriction is not completed by the ring. The tightly constricted young *arc*5 chloroplasts indicate, however, that the lesion in chloroplast division in *arc*5 is caused by a lack of completion of the division process rather than by a retardation in the constriction of the plastid dividing ring. The *arc*5 mutation must therefore affect the final stages of chloroplast constriction, which supports explanation *(i)* above rather than explanation *(ii)*.

The cause of the inability of the *arc*5 chloroplast to complete the division process is presently unclear, therefore we can only speculate. The mutant effect may be caused by a lesion in the fusion of the membranes at the isthmus prior to the separation of the daughter plastids so that separation does not occur before the constant background of chloroplast expansion reverses the constriction of the isthmus as the chloroplast grows in size. Alternatively, the mutation may be a lesion in the construction or function of the plastid dividing ring, possibly by the lack of a vital component so that the ring cannot maintain the constricted state for a sufficient period to allow separation to occur. The isolation and characterisation of the *ARC*5 gene may provide a tool to determine its precise role in the constriction and separation of dividing chloroplasts.

Study of the *arc*5 mutant is also of considerable value for the understanding of the role of two physical factors which affect the accumulation of the chloroplast population - cell size and chloroplast size.

The existence of a period in the very early expansion of wild type mesophyll cells (1000 to 1500 μm^2) during which the proportion of chloroplasts in division rises to a maximal level of 26% before declining suggests a cellular control of the initiation of chloroplast division, as proposed in wheat mesophyll cells by Leech and Pyke (1988). This stimulation, however, does not induce a majority of chloroplasts in the cell to divide together, indicating that division of chloroplasts is not synchronous, even where chloroplast division has been recently initiated. The lack of synchrony is likely to be due to the variation in the size, around the optimum 50μm^2, at which chloroplast division may proceed causing various divisions to be initiated at differing times.

The apparent initial stimulation of chloroplast division is most likely to be a response to the expansion of the cell which, in early development, allows for the expansion of the young chloroplasts to a size at which they are able to divide. Such a control of the initiation of chloroplast division would also indicate that cell size closely restricts expansion and division of young chloroplasts by controlling early cell expansion.

A restriction in the numbers of chloroplasts in division relative to cell expansion and chloroplast accumulation in mesophyll cells supports a theory that chloroplast division is inhibited by the available cellular space so that as the cell expansion proceeds only a small number of chloroplasts are allowed to divide at any time. This effect, combined with chloroplast expansion, would ensure a total chloroplast cover per mesophyll cell which is constantly maintained throughout cell development. This theory is based on the observation that *Arabidopsis* chloroplasts may divide only within a defined range of sizes and that there is a minimum, optimum and maximum size for chloroplast division to be initiated.

An optimum size at which chloroplast division is initiated would infer that a period of expansion is required before young chloroplasts or newly-divided daughter plastids are able to undergo further divisions. Such a period of expansion would explain the initial lag in the numbers of dividing chloroplasts seen in the smaller mesophyll cells in figure 4.7.

The range of possible chloroplast sizes which may permit division at any size between the defined minimum and maximum values would explain why chloroplast divisions in *Arabidopsis* are not synchronous with one another.

The minimal size for chloroplast division in Landsberg *erecta* is similar to the mean chloroplast size of *arc*1 and *arc*7 chloroplasts, suggesting that normal chloroplast division in *arc*1 and *arc*7 mutants is possible at a smaller optimum size than wild type. This is further supported by the reduced optimum size for division of the *arc*1/*arc*5 double mutant compared to wild type.

The largest chloroplast plan areas of the dividing chloroplasts in wild type exceed those of the largest non-dividing chloroplasts, indicating that the largest dividing chloroplasts observed in figure 4.4 are chloroplasts in which division is already underway and which have expanded during division rather than being recently initiated from non-dividing chloroplasts, indicating a maximum size for chloroplast division. This observation suggests that the *arc*5 chloroplast is larger than the size beyond which the completion of chloroplast division is possible. The existence of large chloroplasts in division (4.4) and *arc*5 chloroplasts which have attempted division and subsequently continued to expand shows that chloroplast expansion proceeds during the several chloroplast division cycles. The basis for an optimum size for chloroplast division is not known, but may possibly be due to the structural properties of the chloroplast becoming less stable at 50μm^2. Leech, Thomson and Platt-Aloia (1981) suggest that the initiation of chloroplast division may be a result of an invagination of the central region of the chloroplast following a disturbance in the tensions of the envelope membrane in a similar manner to the theories of Greenspan on cytokinesis (Greenspan, 1977, 1978). This theory was developed in relevance to chloroplast division by Leech, Thomson and Platt-Aloia (1981) and by Possingham and Lawrence (1983). An optimum chloroplast size is consistent with this theory, since smaller chloroplasts will be more stable in size and shape than those of a larger wild type size; the 50μm^2 optimum may be the size at which the chloroplast first begins to become unstable in shape, leading to the initiation of division.

Further work

The study of the co-ordination of chloroplast division by physical phenomena in the mesophyll cell will benefit from the analysis of other mutants, particularly those genotypes in which the co-ordination of chloroplast division is impaired, i.e. *arc*2, *arc*9, *arc*10 and *arc*11. The study of the degree of control of each of the aspects of chloroplast division detailed above will also provide insight into these various mutant effects. The investigation of the optimum size of dividing chloroplasts in *arc*1 and *arc*7 is required to fully authenticate the observations of chloroplast division in the *arc*1/*arc*5 double mutant. An analysis of the co-ordination of the mutant effects such as in *arc*3, *arc*6 and *arc*11 with contrasting mutant effects such as *arc*1 or *arc*7 will enable the effect of these mutations to be observed in an altered genetic background to wild type. The genetic interaction of phenotypically similar mutants, such as *arc*3 and *arc*5 will also provide better understanding of the physical control of chloroplast division as well as an indication of the hierarchy of the *arc* mutant effects in *Arabidopsis*. An analysis of the interaction of *arc*3 and *arc*5 is being undertaken by the construction and isolation of an *arc*1/*arc*3/*arc*5 triple mutant. This triple mutant will enable the interaction of *arc*3 and *arc*5 to be discerned, since the triple mutant will be more easily isolated from the *arc*1/*arc*3 and *arc*1/*arc*5 double mutant parental phenotypes than an *arc*3/*arc*5 double mutant from the similar *arc*3 and *arc*5 mutant phenotypes. The lack of an *arc*1 mutant chloroplast phenotype in the parenchyma sheath cells of the vascular bundle will allow for the analysis of the *arc*3/*arc*5 double mutant effect in those cells of the *arc*1/*arc*3/*arc*5 triple mutant plant.

The study of *arc*5 chloroplast ultrastructure in a series of chloroplasts of increasing size is anticipated to provide more information about the nature of the lesion in chloroplast division of the *arc*5 mutation, and may provide evidence for the size of the chloroplast plan area beyond which the *arc*5 chloroplast loses the potential for division to be completed and suggest theories for the *ARC*5 gene effect and its role in the physical control of chloroplast division.

4.4 SUMMARY

The *arc*5 mutant displays a mesophyll cell mutant phenotype of 14 large chloroplasts the majority of which exhibit a degree of central constriction representative of the early stages of chloroplast division. The *arc*5 mutant chloroplast phenotype suggests that the mesophyll cell chloroplasts have unsuccessfully attempted a chloroplast division and yet can still subsequently expand. The ultrastructure of *arc*5 chloroplasts has been examined to reveal considerable torsion of the thylakoid membranes reminiscent of pre-division thylakoid rearrangement in wild type chloroplasts. The chloroplasts of a double mutant homozygous for the *arc*5 and *arc*1 mutant alleles can divide, showing that the *arc*5 chloroplasts can divide in a different genetic background.

The comparison of the numbers of constricted chloroplasts in Landsberg *erecta* wild type, *arc*5 and the *arc*1/*arc*5 double mutant during mesophyll cell expansion suggests that there is an initial stimulation of chloroplast division in immature mesophyll cells which reduces to a stable number of chloroplasts in division per cell in subsequent cell expansion. Almost all chloroplasts of the *arc*5 mutant demonstrate evidence of attempted division suggesting that all chloroplasts undergo division in the wild type *Arabidopsis* mesophyll cell.

The comparative analysis of the plan areas of chloroplasts in wild type, *arc*5 and the *arc*1/*arc*5 double mutant suggests that in wild type chloroplast division is initiated at an optimum chloroplast plan area of 50μm^2. The analysis of the development of the chloroplast complement in Ler, *arc*5 and the *arc*1/*arc*5 double mutant indicates a tightly co-ordinated physical control of the accumulation and replication chloroplasts in *Arabidopsis*.

CHAPTER 5

The Phenotypic Analysis and Genetic Localisation of *ARC6*

5.1 INTRODUCTION

The ***arc6*** mutant is the most extreme *arc* mutant phenotype yet isolated. The mesophyll cells of the *arc*6 mutant may contain as few as one chloroplast per cell, compared to a WS wild type mean of 80 chloroplasts. The *arc*6 mesophyll cell chloroplasts are up to fifty fold larger than wild type and cover the inner surface of the mesophyll cell in a continuous monolayer in an irregular, lobed shape. The very low chloroplast number of *arc*6 does not increase during cell expansion, indicating that chloroplast division is inhibited. The post-mitotic cells of the *arc*6 mutant also do not accumulate the 14 chloroplasts noted in all other *arc* mutants and wild types, which is almost certainly the number of proplastids apportioned to each cell after mitosis (Pyke and Leech, 1992); suggesting that *arc*6 may also affect proplastid division. *arc*6 is of considerable use to the study of chloroplast division in *Arabidopsis*, since *arc*6 probably shows the maximum extent to which plastid division may be inhibited in a fertile, viable plant. The study of *arc*6 mutant effect is therefore a priority in the study of chloroplast divison in *Arabidopsis*.

The phenotype of the *arc*6 mutant mesophyll cells and whole plant was studied in order to indicate the role of the mutation in the accumulation of chloroplasts in *Arabidopsis*. The analysis confirmed that *arc*6 is a mutation which inhibits proplastid division, and also affects leaf development causing a buckling and twisting of the lamina of primary leaves.

The isolation of the *arc*6 gene by gene tagging has so far proven to be ineffective, since two alleles of the mutant have been isolated, neither of which is tagged with a T-DNA. The alternative approach to gene tagging of a map-based cloning strategy has therefore been initiated by mapping *arc*6 to a chromosomal region of the *Arabidopsis* genome. RFLP mapping was adopted to localise *ARC*6, using the *Arabidopsis* **RFLP Mapping Set (ARMS)**(Fabri and Schäffner, 1994) and **Co-Amplified Polymorphic Sequences (CAPS)** (Konieczny and Ausubel, 1993) RFLP mapping sets, adapted for use with the WS ecotype of *arc*6. The use of these mapping sets has enabled the rapid localisation of *ARC*6 to the central region of chromosome 5. It is anticipated that the localisation of *ARC*6 will aid both the initiation of a chromosome walk to the *ARC*6 locus, as well as facilitating directed mutagenesis using mapped transposable elements such as *Ds* of maize (Feldmann, Malmberg and Dean, 1994).

5.2 RESULTS

5.2.1 The mutant phenotype of *arc*6

(a) The mutant chloroplast phenotype of arc6

The *arc*6 chloroplast phenotype is illustrated in figure 5.1 which shows a mesophyll cell with a single visible chloroplast. The mesophyll cells of *arc*6 usually contain only between 1 and 3 chloroplasts which may be up to 50 fold larger than wild type. When viewed in 3-dimensions by the use of Nomarski differential optics the *arc*6 chloroplast appears to wrap itself around the inner surface of the mesophyll cell in a lobed, but continuous monolayer. The morphology of a single isolated *arc*6 chloroplast released from fixed, broken mesophyll cells compared to wild type chloroplasts is illustrated in figure 5.2. The plasticity of the development of the chloroplast complement by the compensation of chloroplast expansion for perturbations in chloroplast division noted in all *arc* mutants (3.2.2), is particularly evident in *arc*6.

The ultrastructural morphology of *arc*6 chloroplasts was investigated by electron microscopy. Leaf tissue which I embedded was sectioned for electron microscopy by Dr E. Robertson in our laboratory. The *arc*6 chloroplast is located against the cell wall of the mesophyll cell in a thin continuous layer (figure 5.3). *arc*6 chloroplasts are thinner in cross section than the ovoid wild type chloroplasts and may frequently be seen to completely cover other sub-cellular organelles, most notably mitochondria (Pyke, Rutherford, Robertson and Leech, 1994). Neither the thylakoid morphology nor the extent of granal stacking within the *arc*6 chloroplast is significantly different to that of wild type. The normality of the ultrastructure of the *arc*6 chloroplast thylakoid membranes suggests that the extreme size of the *arc*6 chloroplast does not significantly affect the internal chloroplast morphology. A constriction of the *arc*6 chloroplast is sometimes noted, associated with a reduction in the number of thylakoid membranes present at the constriction. Whether this is the result of an aborted attempt at division in the *arc*6 chloroplast, or merely a coincidental fluctuation in the thickness of the chloroplast is not known.

Figure 5.1

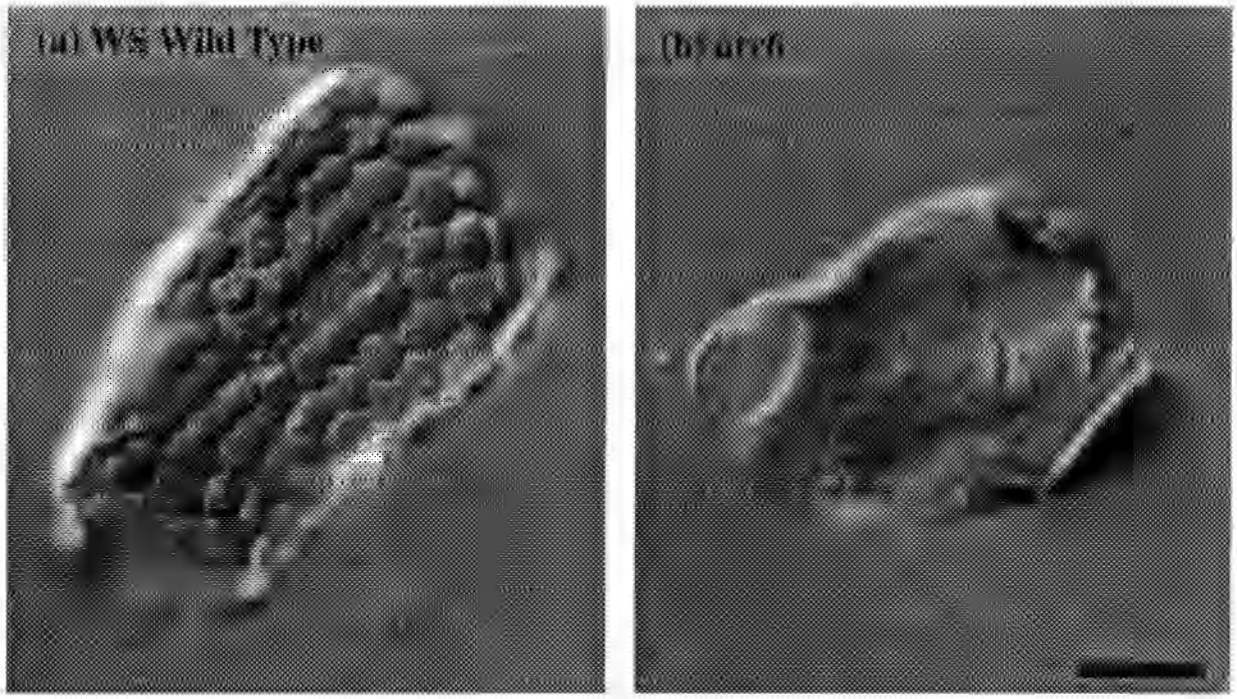


FIGURE 5.1 **WS Wild type and *arc*6 mesophyll cells**

Photomicrograph of isolated mesophyll cells from fully expanded first leaves of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype WS viewed with Nomarski differential contrast optics. (*a*) WS wild type; (*b*) *arc*6. Bar = 25μm.

Reproduced from Pyke, Rutherford, Robertson and Leech, 1994.

Figure 5.2

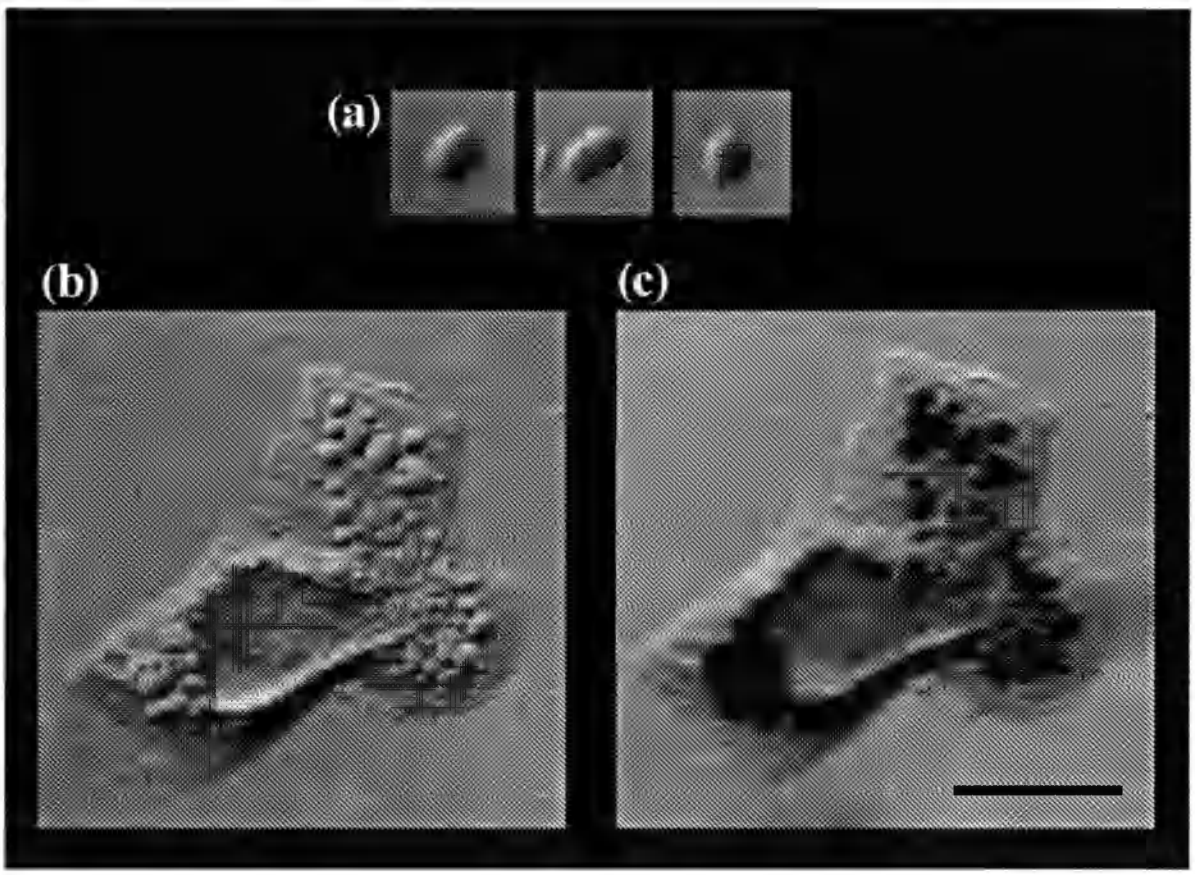


FIGURE 5.2 **WS wild type and *arc*6 isolated chloroplasts**

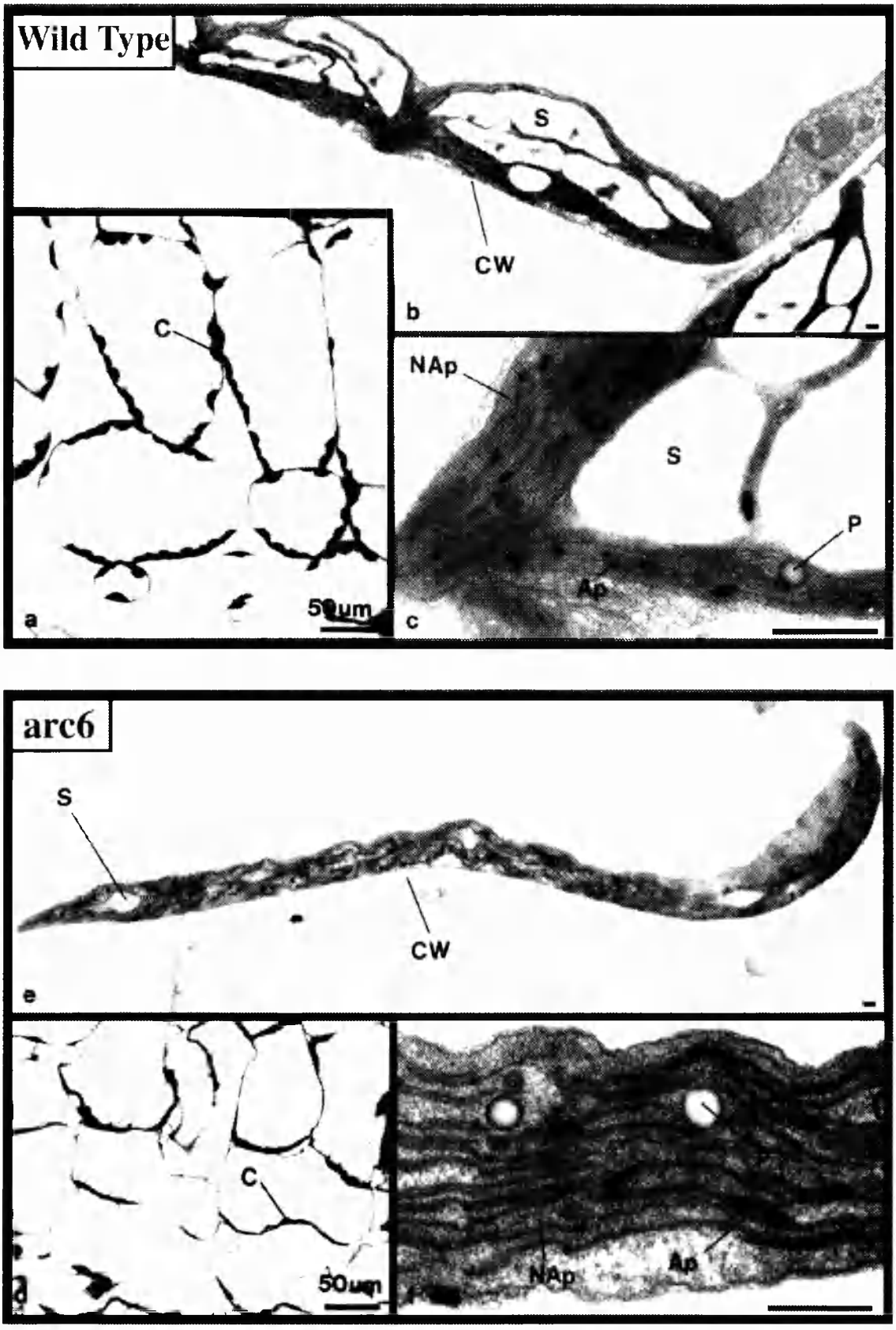
Photomicrograph of isolated mesophyll cell chloroplasts from broken protoplasts of fully expanded first leaves of wild type and *arc*5 mutant of *Arabidopsis thaliana*, ecotype Landsberg *erecta* viewed with Nomarski differential contrast optics. (*a*) WS wild type; (*b*) *arc*6; (*c*) *arc*6 chloroplast stained with iodine to reveal starch grains. Bar = 25µm. Reproduced from Pyke, Rutherford, Robertson and Leech, 1994.

FIGURE 5.3 Ultrastructure of WS wild type and *arc*6 mesophyll cells and chloroplasts

Electron and photomicrographs of resin-embedded sections of mesophyll cells from fully expanded first leaves of wild type and the *arc*6-1 mutant of *Arabidopsis thaliana*, ecotype WS. (*a*) Mesophyll cells of WS wild type indicating distribution of several chloroplasts around the inner cell surface (*b*) Electron micrograph of a portion of a WS wild type mesophyll cell illustrating two adjacent chloroplasts within the cell. (*c*) Electron micrograph illustrating detail of one WS wild type chloroplast. (*d*) Mesophyll cells of *arc*6-1 indicating distribution of few large chloroplasts in a monolayer around the inner cell surfaces of the cells (*e*) Electron micrograph of portion of an *arc*6-1 mesophyll cell illustrating a single chloroplast. (*f*) Electron micrograph illustrating detail of the *arc*6-1 chloroplast. **C**= Chloroplast; **Ap** = Appressed membrane; **NAp** = Non-appressed membrane, **P** = Plastoglobuli; **CW** = Cell wall; **S** = Starch grain. Bar = 500nm.

Reproduced from Pyke, Rutherford, Robertson and Leech, 1994.

Figure 5.3



The distribution of the CtDNA within the wild type and *arc*6 chloroplasts is shown in figure 5.4. The CtDNA, revealed by fluorescence of the DNA stain DAPI in PEG-embedded sections of wild type and *arc*6 leaf tissue (2.3.2-2.3.4), is distributed evenly across the entire chloroplast in a similar distribution to wild type. The distribution of CtDNA across the section of the chloroplast suggests that in *Arabidopsis* the CtDNA is not localised to a discrete region of the plastid, as is observed in wheat (Marrison and Leech, 1992). The *Arabidopsis* CtDNA is therefore either associated with the thylakoid membranes, as is the case in spinach (Rose, 1988) or is distributed throughout the stroma. The ratio of CtDNA to NcDNA per unit of genomic DNA has been noted previously to be similar to wild type (3.3.2). These observations suggest that the CtDNA replication is not significantly perturbed by the complete lack of chloroplast division in the *arc*6 mutant.

The analysis of the number and morphology of *arc*6 proplastids was undertaken in our laboratory by Robertson, Pyke and Leech (1995) during the time when the work for this thesis was being carried out and is included here to aid the discussion of the *arc*6 phenotype. The electron microscopical analysis of sectioned tissue from the shoot and root apical meristems and the leaf primordia of *arc*6 seedlings has revealed considerable disparity between wild type and *arc*6 proplastids (figure 5.5). The *arc*6 proplastid is larger and more irregularly shaped than the wild type proplastid and may be observed in serial sections to wrap around the mitotic nucleus. The *arc*6 proplastid is of an irregular, lobed morphology and is often noted to display constriction of the plastid in various regions. The thylakoid membranes of the *arc*6 proplastid appear more developed than the more rudimentary thylakoids of the wild type proplastid.

(b) The whole plant mutant phenotype of arc6-1 *and* arc6-2

The most prominent mutant feature of the *arc*6 plant is the twisted leaf phenotype (figure 5.6 and 5.7). The *arc*6 leaf is also approximately 25% thinner than the wild type leaf. Other mutant effects include the slightly early senescence of the cotyledons of *arc*6-1 and *arc*6-2 seedlings. The *arc*6 plants also flower approximately 4 days earlier than WS wild type. The *arc*6-1 mutant displays no significant reduction in growth and vigour under growth room conditions and is able to set seed as readily as wild type. *arc*6-2 plants are

FIGURE 5.4 WS wild type and *arc*6 leaf tissue stained for DNA

Photomicrograph of transverse section through PEG-embedded first leaves of *Arabidopsis* WS wild type and *arc*6-1 stained with DAPI and visualised under UV light. The DNA-bound DAPI fluoresces bright blue in the chloroplasts of both WS wild type and *arc*6-1. The light blue fluorescence from the CtDNA is distributed across the section of the plastid in both wild type and mutant chloroplasts. A distortion of mesophyll cell shape is evident in *arc*6-1. C = Chloroplast, each arrow indicates a single chloroplast.

Bar = 25μm.

Figure 5.4

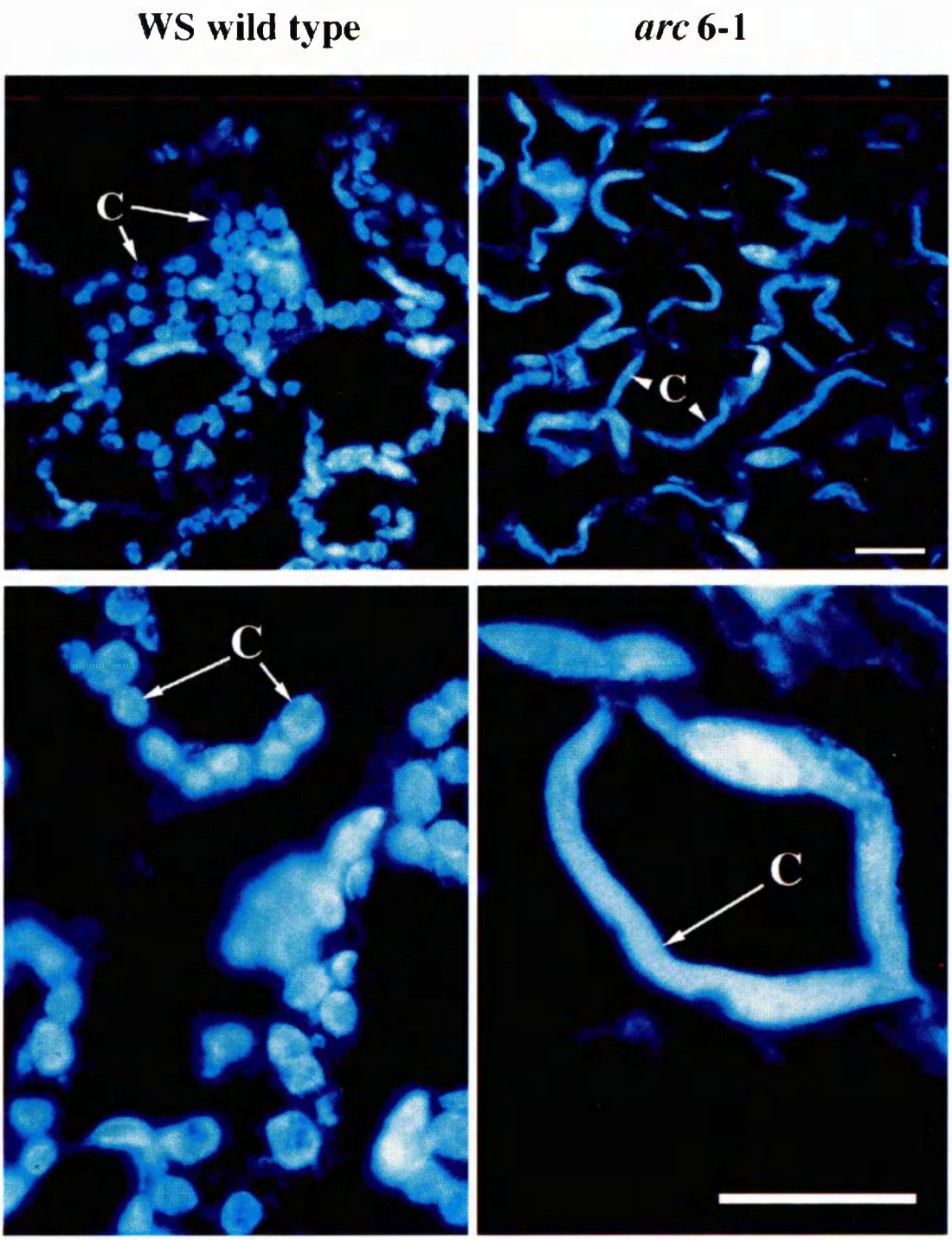


FIGURE 5.5 The ultrastructure of WS wild type and *arc*6 proplastids

Electron micrograph of longitudinal sections (90nm thick) through the *Arabidopsis* shoot apical meristem of (*a*) WS wild type and (*b*) *arc*6-1. Individual proplastids are indicated by arrowheads. The proplastids of *arc*6 are increased significantly in size and display more variable morphology compared to wild type.

N = Nucleus; **CW** = Cell wall; **M** = Mitochondria. Bar = 1μm.

Reproduced from Robertson Pyke and Leech, 1995.

Figure 5.5

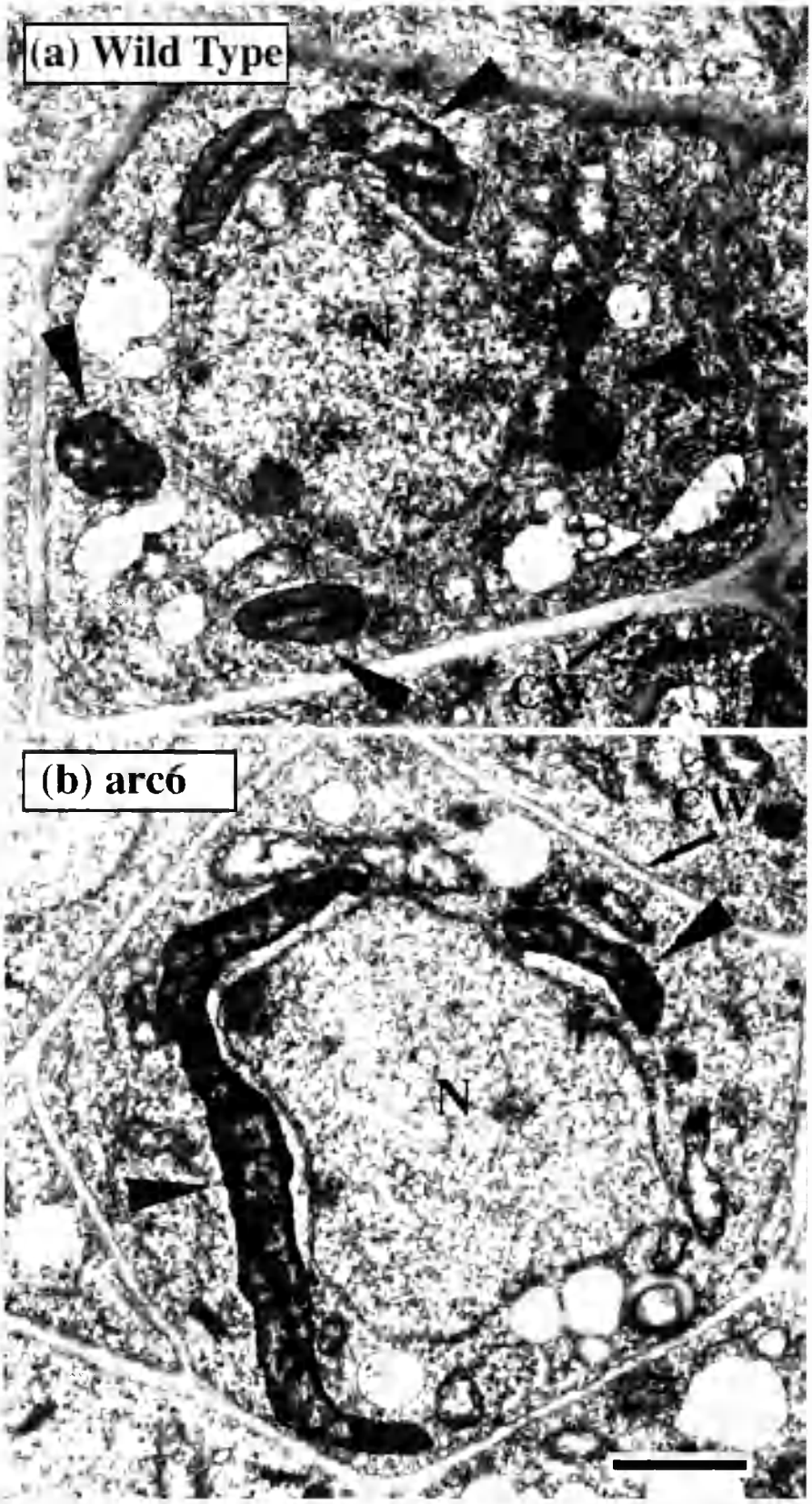


FIGURE 5.6 Seedlings of WS, *arc*6-1 and *arc6-2*

21 day old seedlings of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype WS. (*a*) WS wild type, (*b*) *arc*6-1; (*c*) *arc*6-2. Figures (*b*) and (*c*) exhibit the twisted leaf mutant phenotype of *arc*6. Bar = 10mm

FIGURE 5.7 Mature plants of WS, *arc*6-1 and *arc6-2*

Typical 7 week old plants of wild type and *arc* mutants of *Arabidopsis thaliana*, ecotype WS. (*a*) WS wild type; (*b*) *arc*6-1; (*c*) *arc*6-2. Bar = 50mm.

Figure 5.6

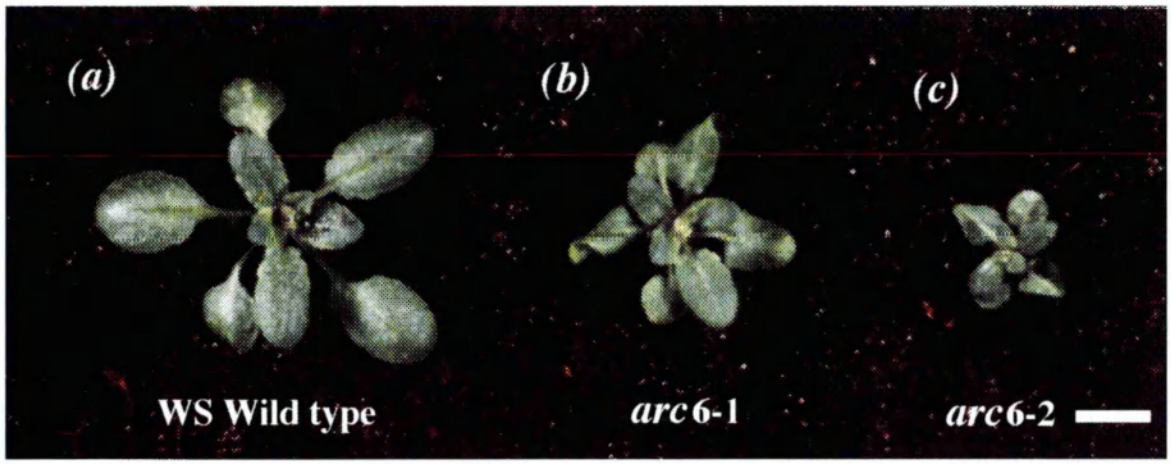
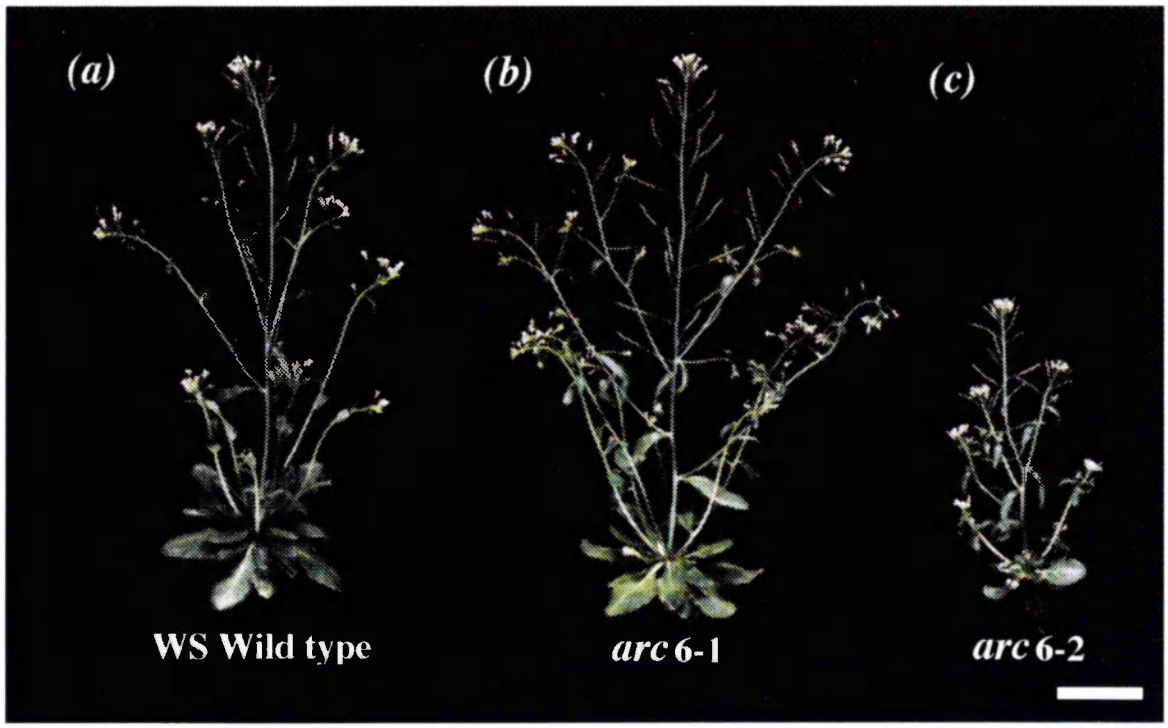


Figure 5.7



stunted in their growth, reaching a mature height of c.150mm, compared to c.300mm in WS wild type and *arc*6-1. The twisted leaf phenotype is apparent in the smaller, less differentiated first leaf; however, it is more extreme in the later primary leaves which are larger than the first leaves in *Arabidopsis*. The ultrastructure of the first leaf of *arc*6-1 was analysed in the transverse section of leaf tissue embedded in Spurr's epoxy resin (2.3.5) and is illustrated in figure 5.8. The mesophyll cells of the *arc*6 leaf are irregular in shape, with jagged and distorted corners to the palisade cells which are a regular, ovoid shape in wild type. The distortion in mesophyll cell shape is also visible in figure 5.5. The palisade and spongy mesophyll cell layers are less distinct in *arc*6 compared to wild type. This contortion of cell shape is also evident in EDTA-digested leaf material (K.A. Pyke, unpublished), suggesting that the altered mesophyll cell shape is not an artefact of the embedding or sectioning process. The epidermal cells also appear to be smaller in *arc*6 leaves than in wild type. The later leaves of *arc*6 plants exhibited a similar alteration in mesophyll cell morphology as in the first leaf.

The seeds of *arc*6 exhibit reduced germination by 20% compared to wild type (figure 5.9) on both sterile growth medium and soil. The reduction in germination frequency for *arc*6 is likely to be a significant factor to the fitness of an *arc*6 mutant plant in conditions of competition. Since *Arabidopsis* is a fast-growing, ruderal plant, any reduction in the rate of germination of the seedling is likely to have a large effect on the overall fitness of the plant. The reason for the reduced germination of *arc*6 plants is not clear since no aborted seeds are obvious in the *arc*6 silique, suggesting that *arc*6 is able to produce apparently viable seed in the parent plant. The reduced germination frequency is therefore more likely to be a lesion in the proces of germination than seed viability.

5.2.2 The genetic localisation of *ARC6*

(a) The cosegregation of arc6 *with the T-DNA insert*

Although neither *arc*6 allele is tagged with a functional T-DNA insert, the truncated T-DNA noted in southern blots of *arc*6-1 DNA (section 3.3.13) would be potentially useful as a probe in subsequent chromosome walking strategies for the isolation of *arc*6 if it mapped sufficiently close to the *arc*6 locus. An analysis of the cosegregation of the T-DNA

FIGURE 5.8 Ultrastructure of WS wild type and *arc*6-1 first leaf

Photomicrograph of a transverse section of resin-embedded (*a*) WS wild type and (*b*) *arc*6 first leaves. Sections stained with toludine blue and sectioned at 1μm thickness. The distorted shape of the *arc*6 mesophyll cells is illustrated in (*b*). The distorted mesophyll cell shape is seen in the *arc*6 cells (indicated by arrows).

C = Chloroplast; **W** = Cell Wall. Bar = 100μm.

Figure 5.8

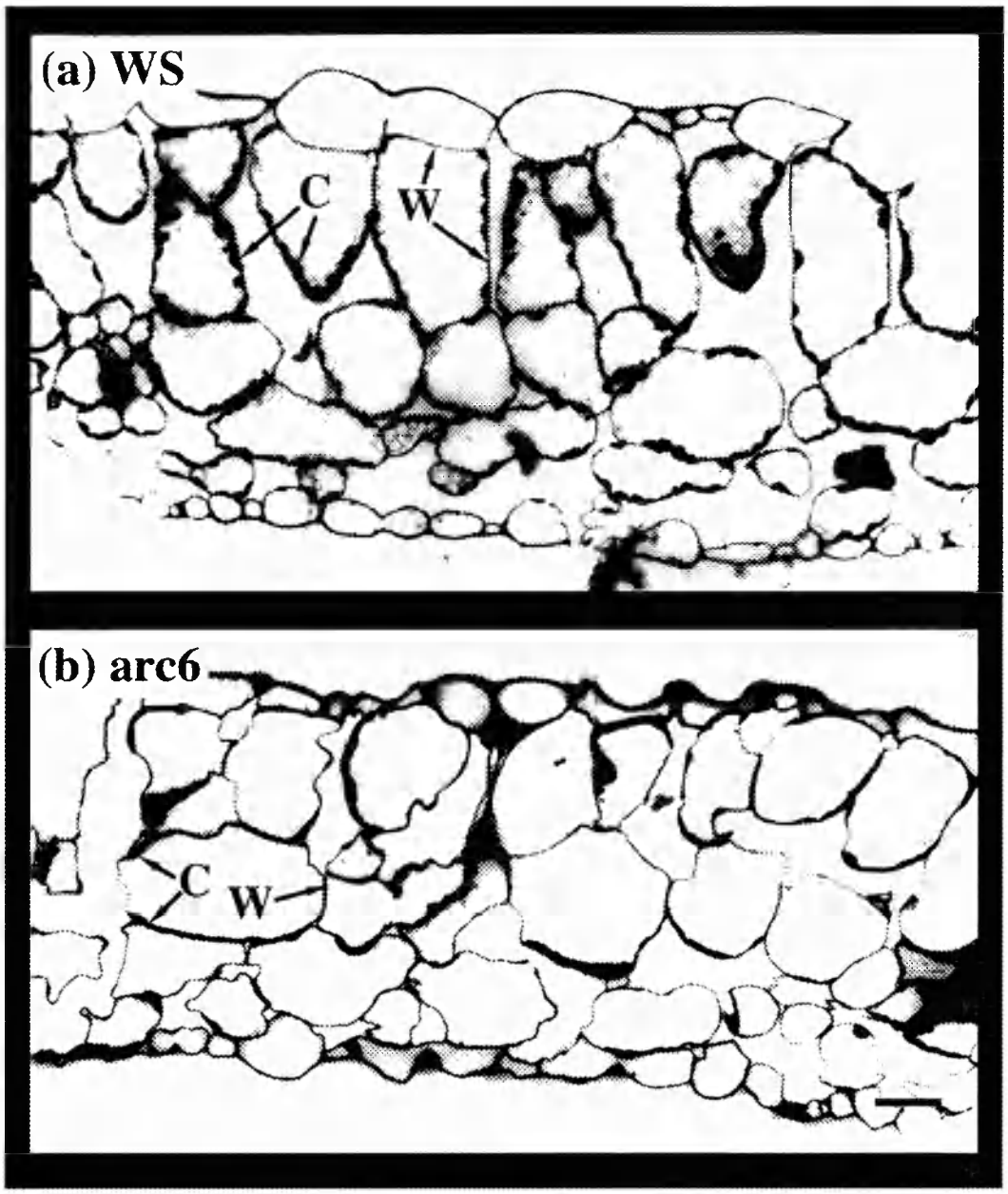
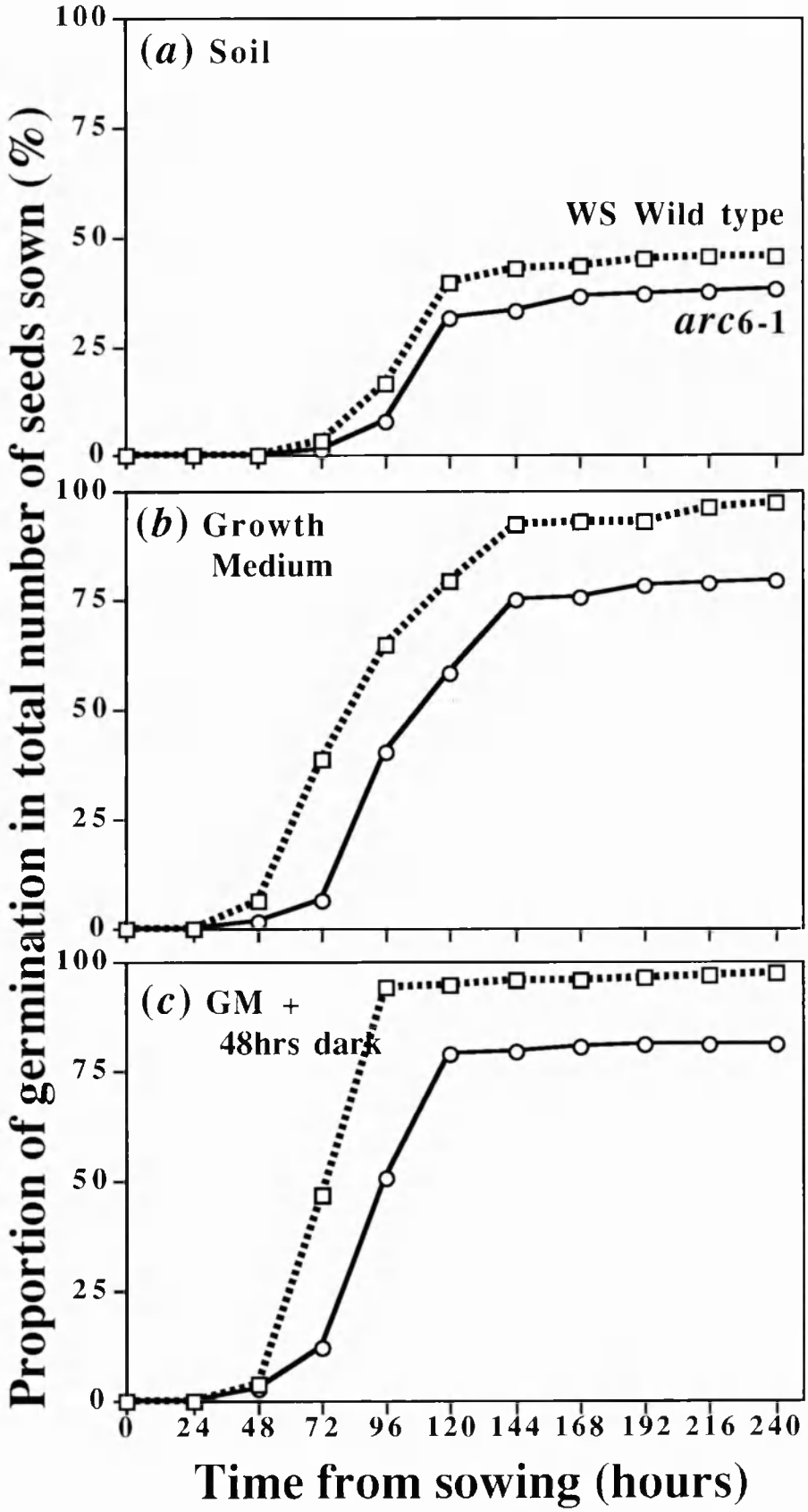


FIGURE 5.9 Germination frequency of WS wild type and *arc*6-1

The relative rates and proportions of the germination of *Arabidopsis thaliana* (ecotype WS) wild type and *arc*6-1 seedlings. (*a*) WS and *arc*6-1 seedlings grown on soil; (*b*) WS and *arc*6-1 seedlings grown on nutrient medium (*c*) WS and *arc*6-1 seedlings grown on nutrient medium, plates placed in dark at 4°C for 48 hours prior to germination. Data obtained from 400 individual seeds sown per genotype per treatment.

Figure 5.9


(a) Soil
WS Wild type
arc6-1
(b) Growth Medium
(c) GM + 48hrs dark
Proportion of germination in total number of seeds sown (%)
100
75
50
25
0
0 24 48 72 96 120 144 168 192 216 240
Time from sowing (hours)

and the *arc*6 locus in *arc*6-1 F_2 mutant sibling DNA from a cross to wild type was therefore undertaken to resolve the genetic distance between the *arc*6 locus and the site of the T-DNA.

The cosegregation of the presence of the T-DNA with the mutant phenotype was carried out on the 42 F_2 mutant sibling DNAs used for the ARMS and CAPS mapping experiments. Identical filters of *Eco*RI-digested DNA used in the ARMS analyses (section 5.3.2*(b)*) were hybridised to the probe to the pBR322 sequences (section 3.3.6) and scored for cosegregation. The pBR322 hybridised to two bands of 7.4kb and 10.5kb length in 31 of 42 individuals (figure 5.10) and is absent in 11 individuals as well as both WS and Landsberg *erecta* wild type lanes. The absence of hybridising T-DNA bands in a mutant sibling indicates the lack of a T-DNA on either chromosome of that individual, i.e. it is fully recombinant. The homozygous or heterozygous nature of the individuals which exhibit hybridisation cannot be determined without further analysis of segregating populations. The *arc*6-1 T-DNA is therefore at least 26.2 cM from the *ARC*6 mutant locus and therefore is not of use as a probe to the *ARC*6 locus in subsequent cloning strategies.

(b) Genetic mapping of ARC6

T-DNA tagging to facilitate the isolation of the *ARC*6 gene has proven to date to be unproductive. This is because the genome of the *arc*6-1 allele contains a right border truncated T-DNA that is not linked to the *arc*6 locus and the T-DNA present in the genome of the *arc*6-2 allele is too severely truncated to be of use in gene isolation. Other T-DNA sequences may be present in the *arc*6-1 or *arc*6-2 genomes, but since these are not detectable by the use of right and left border probes, they are not of use for gene isolation by plasmid rescue (Castle, Errampalli, Atherton *et al*, 1993).

Since the *ARC*6 locus may not be isolated from the two available alleles by gene tagging, the isolation of the *ARC*6 locus is therefore being undertaken by two complementary approaches: *(i)* the search for further tagged mutant alleles of the locus, and *(ii)* the initiation of a map-based cloning strategy.

(i) Despite recent advances in the genetic mapping of *Arabidopsis*, the use of the technique of chromosome walking for the isolation of the *ARC*6 gene is not anticipated to

Figure 5.10

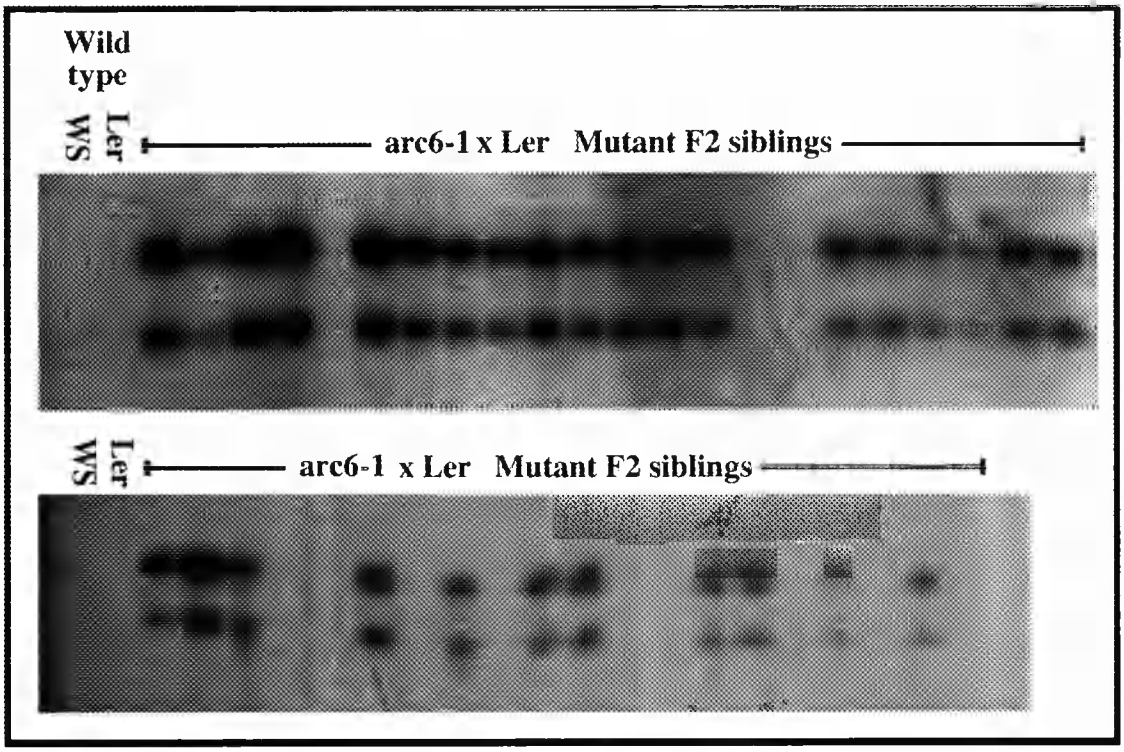


FIGURE 5.10 Cosegregation of *arc*6-1 with the T-DNA

Autoradiograph of a Southern blot of *arc*6-1/L.*er* F_2 mutant sibling DNA indicating the cosegregation of the T-DNA with the *arc*6-1 mutant locus. F_2 sibling genomic DNA was cut with *Eco*RI restriction enzyme and probed with ^{32}P-labelled pBR322 sequence. The pBR322 probe recognises two fragments of the T-DNA but does not cosegregate with the *arc*6-1 mutant phenotype suggesting a low degree of linkage between the two loci.

be as efficient as the use of gene tagging. There is a reasonable expectation that a screen for more alleles of the *arc*6 mutant in the T-DNA or transposon-mutagenised populations of *Arabidopsis* will eventually yield a tagged allele which may then be used for the isolation of the *ARC*6 locus. The further screening of mutagenised lines for tagged alleles of *arc*6 will be greatly aided by the twisted leaf mutant plant phenotype enabling plants to be screened by eye rather than by scoring cellular phenotypes under the microscope.

(ii) In the absence of a tagged *arc*6 allele at present, the initiation of a map-based cloning strategy is anticipated to facilitate the isolation of the *ARC*6 locus in the long term by the use of chromosome walking techniques (Gibson and Somerville, 1992). The recent advances in the characterisation of the *Arabidopsis* genome as part of the international *Arabidopsis* genome project should aid the isolation of a mapped locus. The development of contiguous regions of mapped YAC and Cosmid clones of *Arabidopsis* genomic DNA is also anticipated to aid the map-based cloning of a finely mapped locus (Hwang, Kohchi, Hauge *et al*, 1991; Schmidt, Cnops, Bancroft and Dean, 1992; Schmidt and Dean, 1992 and 1993). These contiguous regions of YAC and cosmid clones, however, do not yet cover very large areas of the *Arabidopsis* genetic map, although the YAC cover of chromosomes 4 is complete (Schmidt, West, Love *et al*, 1995) and 5 has approximately 50% cover (R. Schmidt, personal communication). The localisation of the *ARC*6 locus to a well characterised region of the *Arabidopsis* genetic map would increase the potential for the isolation of the locus by chromosome walking. The localisation of *ARC*6 would also facilitate the production of new *arc*6 alleles by site-directed mutagenesis using mapped transposable elements (Walbot, 1992; Feldmann, Malmberg and Dean, 1994) and may potentially provide tagged alleles of *arc*6 in the near future. Provided that the *ARC*6 locus is reliably localised to a region of the *Arabidopsis* genetic map which is near to such a transposable element, the element could be induced to transpose and may potentially produce new *arc*6 mutant alleles tagged with a transposon. The localisation of *ARC*6 to the *Arabidopsis* genetic map may also prove advantageous in other unrelated areas of *Arabidopsis* research. The location of another characterised morphological mutant phenotype of *Arabidopsis* to the genetic map will increase the density of genetic markers available to other researchers, especially those investigating genes in the region near to the

*ARC*6 locus. It will also aid in the definition of the several *Arabidopsis* genetic maps such as the Lister and Dean Recombinant Inbred integrated map (Lister and Dean, 1993).

The identification of a map position for the *ARC*6 locus could be achieved in several ways: Genetic mapping using morphological markers; RFLP mapping using the *Arabidopsis* **R**FLP **M**apping **S**et (**ARMS**) (Fabri and Schäffner, 1994); RFLP mapping using **C**o-**a**mplified **P**olymorphic **S**equences (**CAPS**) (Konieczny and Ausubel, 1993); or RFLP mapping using **R**andom **A**mplified **P**olymorphic **S**equences (**RAPD**) (Reiter, Williams, Feldmann *et al*, 1992).

The efficiency of conventional genetic mapping involving the analysis of the cosegregation of the mutant phenotype with one of several mapped morphological and physical markers has been greatly advanced by the production of multiple marker lines, such as W100 (Koornneef, Hanhart, van Loenen-Martinet, Peeters and van der Veen, 1987). The marker line W100 is homozygous for nine independent mutations located across the *Arabidopsis* genome. Outcrossing the mutant allele of interest to the marker line facilitates mapping of the mutation to the chromosomal region of one of the nine morphological markers (Koornneef and Stam, 1992). Once a general location of the locus of interest has been determined, mapping using marker lines which are homozygous for a single morphological marker which maps close to the mapped *arc*6 locus will be adopted to verify the genetic map position of the gene. However the approach of using morphological markers requires the scoring of very large numbers of progeny of the crosses between the mutant and the marker line and is extremely labour-intensive.

As an alternative to using morphological markers, I chose the technique of RFLP mapping to localise the *ARC*6 gene. RFLP mapping of the *arc*6 mutant locus can be a relatively precise approach, but would be a very long-term approach unless a multiple-marker mapping strategy is adopted (Fabri and Schäffner, 1994). The use of multiple marker sets avoids the requirement to test individual RFLP markers for polymorphisms as each new marker is adopted. The **ARMS** and **CAPS** mapping systems have recently been developed to allow the simultaneous mapping of several markers on a single backcrossed mutant population. The ARMS and CAPS systems were adopted for the

localisation of *ARC*6. The ARMS system of RFLP mapping using Southern blot analysis was adopted for the initial localisation of the *arc*6 mutant locus since the PCR techniques required for CAPS mapping were not initially accessible. The CAPS markers were used subsequently to verify the map position of *ARC*6 proposed by the ARMS analysis.

(i) Mapping by the Arabidopsis ***RFLP Mapping Set (ARMS)*** *indicated linkage of* ARC6 *to marker* ***m247*** *on chromosome* ***5***

The ARMS mapping system was developed by Fabri and Schäffner (1994) to facilitate the mapping of untagged mutations in *Arabidopsis* The ARMS markers were adapted from existing RFLP markers of known map positions. The markers were selected to provide an even spread of 31 markers across the *Arabidopsis* genome at a distance of not more than 50 cM apart. The ARMS markers were also selected for those which identified a polymorphism between *Arabidopsis* ecotypes Landsberg *erecta* and Columbia with a single restriction enzyme digest using *Eco*RI. Provided that the resolution of the polymorphisms was sufficient, several markers could then be used simultaneously to probe a single *Eco*RI-digested Southern blot of several genomic DNA samples from mutant F_2 progeny of a cross between Landsberg *erecta* and Columbia ecotypes. Cosegregation of the polymorphism ecotype recognised by the ARMS marker with that of the mutation would indicate linkage between the two loci. A map location of the gene could therefore potentially be obtained in only two hybridisations using cocktails containing *several* ARMS probes at once, as opposed to several consecutive hybridisations with single probes required in conventional RFLP analysis. The spread of markers across the genome is sufficiently compact to indicate potential linkage between a mutant locus and a single marker with only a small population of 20 mutant individuals (Fabri and Schäffner, 1994). Further cosegregation analysis with more individuals or additional markers from the region indicated by the mapping set would provide a greater resolution indicating more significant linkage for subsequent RFLP analysis of the locus.

The ARMS system provides an effective means of localising mutations with a small number of mutant siblings, however the system was designed for use between Landsberg *erecta* and either Columbia or Enkheim ecotypes. The identification of

polymorphisms between Ler and WS revealed by the ARMS probes was required before the ARMS system could be applied to mapping the *arc*6 mutation.

The spread of ARMS markers across the *Arabidopsis* genome is illustrated in Figure 5.11. In order for the ARMS system to be used to map the *arc*6-1 mutation (WS ecotype) backcrossed to Ler wild type, the ARMS markers were tested for polymorphism between the ecotypes Landsberg *erecta*, WS and Columbia. Table 5.1 summarises the polymorphisms identified between these ecotypes. 19 of the 25 ARMS markers tested exhibited a polymorphism between Ler and WS and the spread of these markers, which show WS/Ler polymorphisms, across the *Arabidopsis* genome is illustrated by Figure 5.12. An initial mapping set was constructed for the localisation of *ARC*6 by hybridisation to the DNA of 22 F_2 mutant progeny of a cross between *arc*6-1 (WS ecotype) and Landsberg *erecta*. However, difficulty in scoring a large set of several ARMS markers prompted the use of the ARMS probes either individually or in pairs. This technique was still advantageous to the normal techniques of RFLP analysis since the same *Eco*RI-digested Southern filter could be used for all the ARMS probes rather than requiring a new filter of DNA digested with a different enzyme for each individual RFLP probe.

DNA samples from 22 mutant F_2 siblings of a cross between *arc*6-1 and Landsberg *erecta* were used to localise the *arc*6-1 mutation to one of the five chromosomes. 8 ARMS markers were tested against *arc*6; the only marker which showed cosegregation to the *ARC*6 locus was m247 (table 5.2). m247 maps to the central region of chromosome 5 (figure 5.11); cosegregation of *arc*6-1 to m247 would therefore indicate that *ARC*6 was located on chromosome 5. Only 6 of the 22 mutant F_2 siblings displayed a recombinant *heterozygote* phenotype when probed with the m247 probe (figure 5.13). The heterozygous individuals each therefore contained one recombinant chromosome (shown by the Ler polymorphism) and one non-recombinant WS chromosome. Therefore, only *6* of the 44 chromosomes tested (2 chromosomes from each of 22 mutant individuals) displayed recombination between the m247 and *ARC*6 loci. This low proportion of recombinants (a recombination frequency of 13.6%) indicated that *ARC*6 mapped to within 14cM of m247. The marker m247 is located at approximately the middle of chromosome 5 (figure 5.11), suggesting that ***ARC*6 maps to chromosome 5.**

FIGURE 5.11 RFLP map of *Arabidopsis* showing ARMS and CAPS markers

RFLP map of *Arabidopsis thaliana* indicating the location of ARMS and CAPS RFLP markers on the five chromosomes. The location of the *erecta* mutation (present in Ler ecotype) is also indicated. Bar = 10cM.

Figure 5.11

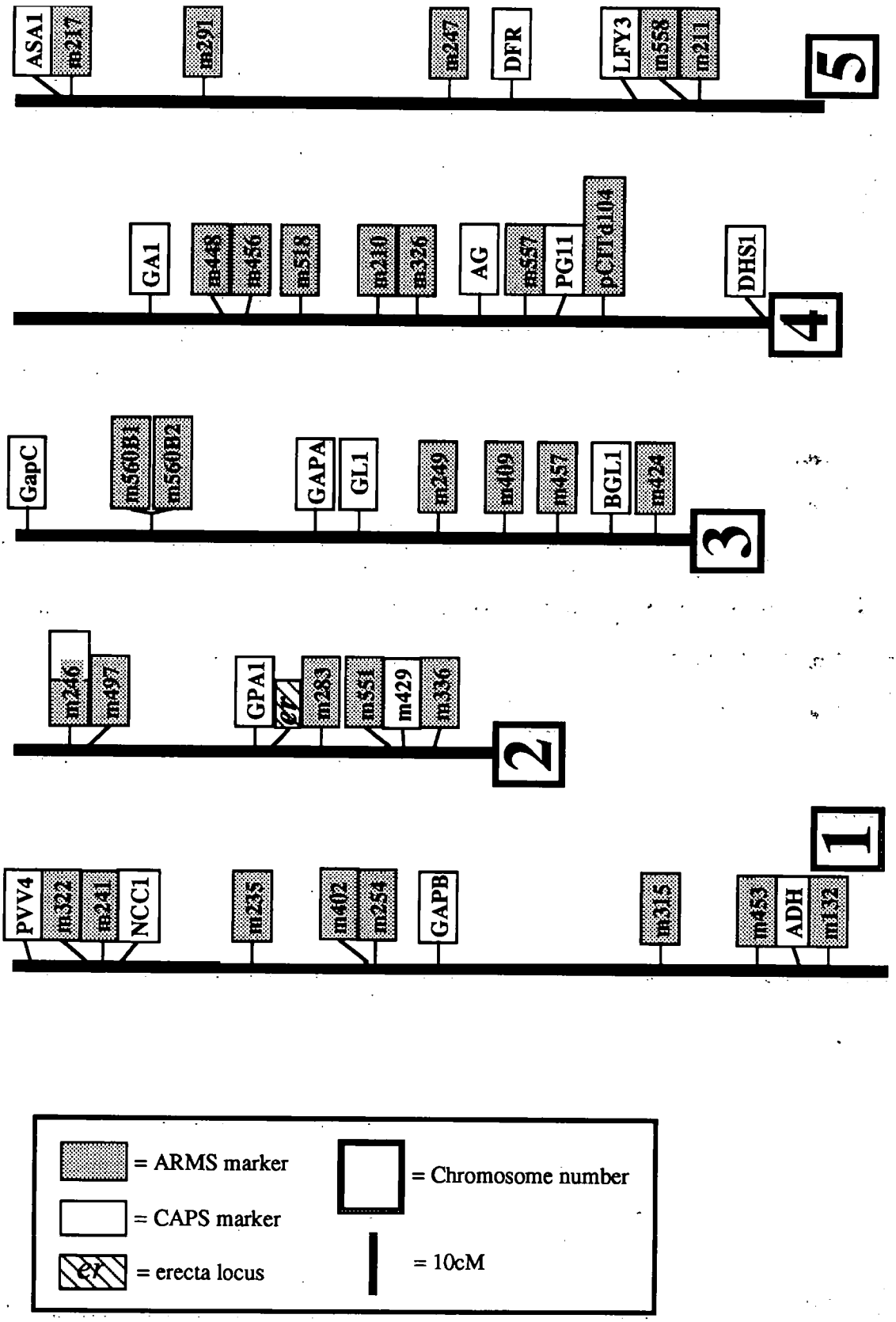

ASA1
m217
m291
m247
DFR
LFY3
m558
m211
5
GA1
m448
m456
m518
m230
m326
AG
m557
PG11
pCT1d104
DHS1
4
GapC
m560B1
m561B2
GAPA
GL1
m249
m409
m457
BGL1
m424
3
m246
m497
GPA1
er
m283
m551
m429
m336
2
PVV4
m322
m241
NCC1
m235
m402
m254
GAPB
m315
m453
ADH
m132
1
= ARMS marker
= CAPS marker
er = erecta locus
= Chromosome number
= 10cM

Table 5.1 WS/Ler/Col polymorphisms of ARMS markers

Table of RFLP polymorphisms with ARMS markers between Landsberg *erecta*, WS and Columbia ecotypes of *Arabidopsis thaliana*. Genomic DNA was cut with *Eco*RI restriction enzyme. RFLP fragment sizes are in kilobase pairs. Chromosomal location of ARMS markers is indicated, derived from C. Lister (personal communication).

ARMS Marker	Chromosome number and marker location	L.er (*Eco*RI digest) (kb)	WS (*Eco*RI digest) (kb)	Col (*Eco*RI digest) (kb)
m322 A	1 : 15.0	10.8	11.5	10.8
m241 A	1 : 16.6	4.33	4.67	5.2
m235 A	1 : 48.7	11.6	11.6	10.4
m402 A	1 : 56.0	9.0	7.8	7.8
m254 A	1 : 67.6	5.3	4.67	4.67
m315 B	1 : 120.3	2.7	2.7	7.8
m453 A	1 : 138.0	7.5	7.5	2.96
m132 A	1 : 151.1	5.3	5.3	7.2
m246 B	2 : 10.8	6.2	2.16	2.16
m497 A	2 : 13.4	6.5	6.5	4.46
m283 C	2 : 58.9	2.55		2.34
m551 C	2 : 73.5	3.02	8.5	3.02
m336 A	2 : 78.3	8.7	9.9	9.9
m560 B1	3 : 35.5	3.1	1.7	1.7
m560 B2	3 : 35.5	3.1	1.6	1.6
m429 A	3 : 73.5	6.6	5.7	5.7
m409 B	3 : 70.4	3.48	3.8	3.8
m457 A	3 : 100.3	12.3	12.3	9.3
m424 A	3 : 118.9	5.3	4.58	4.58
m456 A	4 : 18.1	4.47	3.61	3.61
m448 A	4 : 38.4	7.1	8.9	8.9
m518 A	4 : 53.4	10.8	8.1	8.1
m210 A	4 : 60.0	8.7		
m326 B	4 : 75.6	3.54	4.24	4.24
m557 A	4 : 85.0	9.7	9.7	9.7
d104 C	4 : 109.1	4.78	2.51	2.51
m217 C	5 : 10.5	2.02	1.76	1.76
m291 C	5 : 34.5	2.2	2.1	2.1
m247 A	5 : 80.8	8.1	12.7	12.7
m558 A	5 : 92.0	11.2	8.8	8.8
m211 A	5 : 127.1	4.55		4.9

FIGURE 5.12 RFLP map of WS/Ler polymorphic ARMS and CAPS markers

RFLP map of *Arabidopsis thaliana* indicating the location of ARMS and CAPS RFLP markers on the five chromosomes which exhibit polymorphisms between WS and Ler ecotypes. ARMS markers not yet tested for polymorphism between WS and Ler are also indicated in smaller font size. Bar = 10cM.

Figure 5.12

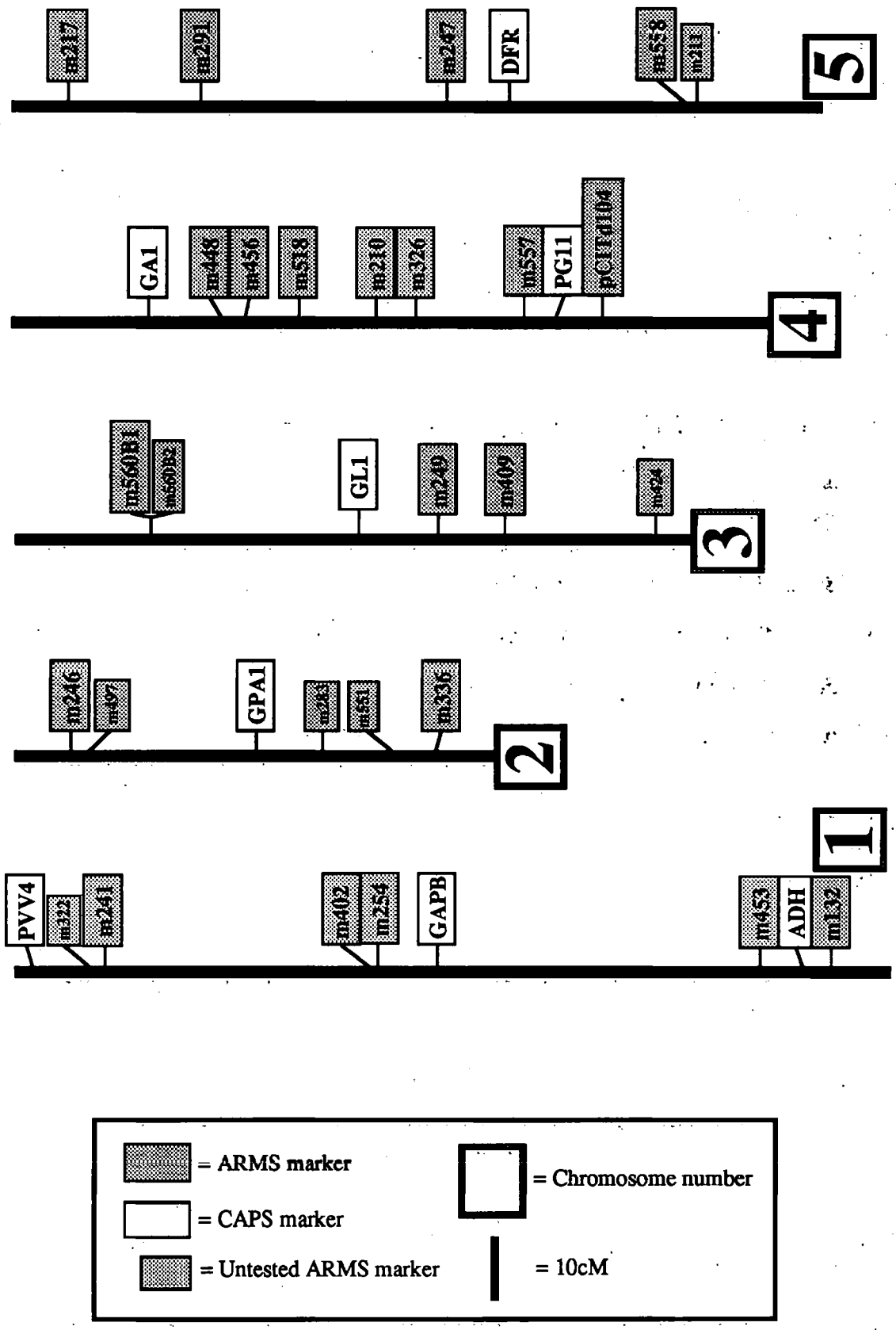

m317
m201
m247
DFR
m558
m213
5
GA1
m448
m456
m538
m210
m326
m557
PG11
pCIT1104
4
m564R1
m5013
GL1
m249
m409
m24
3
m246
m497
GPA1
m243
m481
m336
2
PVV4
m322
m241
m402
m254
GAPB
m453
ADH
m132
1
= ARMS marker
= CAPS marker
= Untested ARMS marker
= Chromosome number
= 10cM

Table 5.2 Cosegregation of *arc*6-1 with ARMS and CAPS markers

Table indicating the results of ARMS and CAPS mapping analyses of *arc*6-1. 42 *arc*6-1/L.*er* F_2 mutant sibling DNAs are numbered between 1 and 71 and the genotype of each marker locus is indicated for each sample. **W** = WS genotype; **L** = L.*er* genotype; **H** = Heterozygote; **X** = either WS or Heterozygote; **Y** = either L.*er* or Heterozygote; **U** = Unscorable. The number of recombinant (**R**) chromosomes exhibiting L.*er* genotype and the number of non-recombinant (**NR**) chromosomes exhibiting WS genotype is included.

MAPPING SET	MARKER	CHR	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	24	25
ARMS	m241	1					H	L				W				H		H	H	L	W			H	L
ARMS	m254	1					L	H				W				H		W	L	L	H			W	H
ARMS	m246	2					W	L				W				L		L	W	H	H			W	H
ARMS	m336	2					H	H				H				L		H	L	H	H			H	W
ARMS	d104	4					Y	Y				Y				W		Y	U	W	Y			Y	Y
ARMS	m217	5					U	U				L				L		W	H	H	H			H	W
ARMS	m247	5					H	W				W				H		W	H	W	W			W	W
ARMS	m558	5					W	U				H				U		X	U	L	U			U	U
CAPS	GPA1	2	W	W	H	W	W	H	Y	W	W	W	H	L	W	L	L	U	U	W	L	L	L	L	U
CAPS	GA1	4	W	W	L	U	W	W	H	H	H	W	H	L	L	W	H	W	W	L	W	W	L	H	L
CAPS	DFR	5	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W

MAPPING SET	MARKER	CHR	26	34	37	39	40	42	43	44	45	48	49	50	52	53	54	66	67	68	69	71	R	NR
ARMS	m241	1		L		H	L	H					H	H	L			H	H	W	H	H	25	19
ARMS	m254	1		W		H	H	L					U	L	H			H	W	U	U	U	18	18
ARMS	m246	2		W		H	H	W					H	L	H			H	W	W	W	L	18	26
ARMS	m336	2		L		H	W	H					H	H	H			W	H	L	L	H	24	20
ARMS	md104	4		Y		Y	W	W					H	Y	Y			Y	Y	U	U	U	20.5	15.5
ARMS	m217	5		H		H	H	H					U	W	L			H	H	H	L	L	21	17
ARMS	m247	5		W		H	W	W					W	W	W			H	H	W	W	W	6	38
ARMS	m558	5		U		H	L	H					W	L	L			H	H	H	U	L	16.5	11.5
CAPS	GPA1	2	W	W	L	H	X	H	H	W	H	L	L	L	L	H	L	L	W	U	W	L	39	45
CAPS	GA1	4	H	H	H	L	H	H	W	W	L	H	H	H	L	H	L	H	H	W	H	H	38	38
CAPS	DFR	5	L	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	W	H	3	81

Figure 5.13

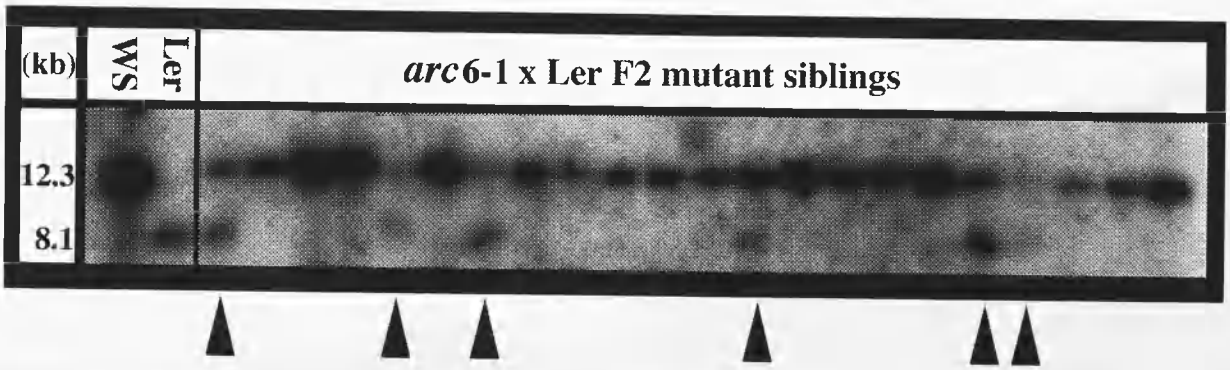


FIGURE 5.13 **Cosegregation of *arc*6-1 with m247 (ARMS)**

Autoradiograph of a Southern blot of *arc*6-1/Ler F_2 mutant sibling DNA indicating the cosegregation of the *arc*6-1 mutant locus with the ARMS marker **m247**. F_2 sibling genomic DNA was cut with *Eco*RI restriction enzyme and probed with ^{32}P-labelled m247 which recognises a 12.3 and 8.8 kb fragment in WS and Ler genotypes respectively. Heterozygous samples are indicated by arrows.

The location of *ARC6* on chromosome 5 was verified by analysis with the CAPS marker DFR, since there were no more ARMS markers in the region of m247 which displayed polymorphisms between WS and Ler. The cosegregation of *arc6*-1 with DFR would confirm that *ARC6* mapped to the central region of chromosome 5.

(ii) Mapping by Co-Amplified Polymorphic Sequences (CAPS) indicated linkage of ARC6 *to the marker* **DFR** *on chromosome 5*

The CAPS system of mapping *Arabidopsis* mutations was developed by Konieczny and Ausubel (1993). The nucleotide sequences of 18 mapped genes or RFLP loci, distributed across the five chromosomes in an even spread, were used to synthesise primers for the amplification of fragments of those loci by PCR. The amplified sequences were then tested with several restriction enzymes to reveal restriction fragment length polymorphisms between Landsberg *erecta* and Columbia ecotypes. Once a polymorphism was established for the PCR product of each CAPS primer, the CAPS primers could then used individually to amplify a single fragment of DNA from genomic DNA of several mutant F_2 siblings of a Ler/Col cross which could be tested by a restriction digest to reveal the Ler or Columbia ecotype of the chromosomal DNA at the CAPS marker locus. Significant identity of the ecotype of the DNA fragment amplified by one of the CAPS primers to that of the mutation would indicate linkage of the mutant locus to the CAPS marker locus and suggest a map position for the mutant gene.

The CAPS system was designed primarily for use with Ler and Col ecotypes. The CAPS markers were tested for polymorphisms shown in the WS ecotype by J. Chandler and A. Wilson (A. Wilson, personal communication). 8 of the 18 CAPS markers were found to amplify sequences which were polymorphic between Ler and WS (table 5.3), and which could therefore be used with the *arc6*-1 x Ler backcrossed material with which the ARMS mapping was carried out. The use of the CAPS system increased the accuracy of the ARMS mapping system in the mapping of *arc6* by providing an extra marker in the region of the genome indicated by the ARMS mapping of the *ARC6* locus.

The CAPS marker **DFR** is localised in the region of ARMS marker m247, and was therefore used for the cosegregation analysis with the *arc6*-1 mutation. The CAPS

Table 5.3 WS/Ler/Col polymorphisms with CAPS markers

Table of RFLP polymorphisms with CAPS markers between Landsberg *erecta*, WS and Columbia ecotypes of *Arabidopsis thaliana*. CAPS PCR fragment sizes are in base pairs. Chromosomal location of ARMS markers is indicated, derived from C. Lister. RFLP information from data of A.Wilson and J. Chandler (personal communication).

CAPS Marker	Chromosome number and marker location (cM)	Restriction enzyme	WS bands look like
PV4	1 : 3.7	*Bsa*AI	Columbia
NCC1	1 : 19.1	*Rsa*I	Landsberg
GAPB	1 : 79.3	*Dde*I	Columbia
ADH	1 : 146.8	XbaI Sau3AI	Columbia Landsberg
m246	2 : 10.8	*Mae*III	Landsberg
GPA1	2 : 45.1	*Afl*III	Columbia
m429	2 : 73.5	*Scr*FI	Landsberg
GAPC	3 : 3.0	*Eco*RV	Landsberg
GAPA	3 : 56.6	*Dde*I	Landsberg
GL1	3 : 64.1	*Taq*I	NOVEL PATTERN
BGL1	3 : 111.2	Sau3AI RsaI	Landsberg Landsberg
GA1	4 : 25.9	*Bsa*BI	Columbia
AG	4 : 86.7	*Xba*I	Landsberg
PG11	4 : 100.4	*Bfa*I	Columbia
DHS1	4 : 139.3	*Dde*I	Landsberg
ASA1	5 : 9.0	*Bcl*I	Landsberg
DFR	5 : 92.0	*Bsa*AI	Columbia
LFY3	5 : 115.3	*Rsa*I	Landsberg

experiment was also carried out on markers GA1 and GLA1 to enhance the cover of mapping markers across the *arc*6 genome to verify that *arc*6 did not appear to be localised to any other chromosomal regions. The CAPS experiments were carried out on DNA from a population of 42 mutant F_2 siblings of an *arc*6-1 x Ler backcross. 40 of the DNA samples exhibited a WS ecotype in the DNA fragment amplified by the DFR probe. Only **ONE** of the F_2 sibling DNA samples displayed a *Ler* polymorphism, and *one* DNA sample was *heterozygous* at the DFR locus for Ler and WS (Figure 5.14, Table 5.2). Only *3* of the 84 chromosomes (2 from each of 42 mutant individuals) tested were therefore recombinant between DFR and *ARC*6, indicating a genetic distance of *c.*3.6cM.

The cosegregation of DFR with *arc*6-1 confirms the initial indication of the ARMS mapping that ***ARC*6 maps to the central region of chromosome 5.**

The *arc*6 mutation maps very close to DFR and was likely to be located either between DFR and m247 or on the distal side of DFR to m247. The mapping data were applied to the Mapmaker mapping programme (Lander, Green, Abrahamson, *et al*, 1987), which predicts, using the *Kosambi* mapping function (Kosambi, 1944; Koornneef and Stam, 1992), the most probable linkage between the markers tested and the most likely orientation for the mapped locus relative to the markers with which it cosegregates. The Mapmaker data suggested that the most probable location for *ARC*6 was **between** the markers **m247** and **DFR**. The predicted position for the *ARC*6 locus is dependent on the data of only a small sample of F_2 mutant individuals so that the genetic distance predicted between markers is not fully reliable. The comparison of the suggested map position of *ARC*6 to the data of the physical map developed from the recombinant inbred lines of Lister and Dean (1993) provided a more accurate estimation of the location of *ARC*6. The comparison of the genetic data provided by the RFLP analysis with the RI genetic map was facilitated by the *Joinmap* computer mapping programme (Stam, 1993) indicated that *ARC*6 probably is located between the two markers, **17.2 cM** from **m247** and **5.1 cM** from **DFR**. The location of *ARC*6 at the central region of chromosome 5 is shown on a *physical* map of chromosome 5 (figure 5.15) prepared from data donated in March 1995 by Dr C. Lister which was derived from mapping analyses of several mutations and RFLP probes to the Recombinant Inbred lines (Lister and Dean, 1993).

FIGURE 5.14 Cosegregation of *arc*6-1 with DFR (CAPS)

Photograph of agarose gel containing *Bsa*AI-digested PCR fragments amplified from *arc*6-1 / Ler F_2 mutant sibling DNA using the DFR CAPS primer. DNA was visualised by staining with ethidium bromide and fluoresced under UV light. WS genotype DNA is restricted to 609 and 534 bp fragments; L.*er* genotype DNA is restricted to 609, 525, 318 and 216 bp fragments. Sample 26 is L.*er* genotype, sample 71 is heterozygous for WS and L.*er*; all other samples are WS genotype. Size markers of 100bp intervals are indicated.

Figure 5.14

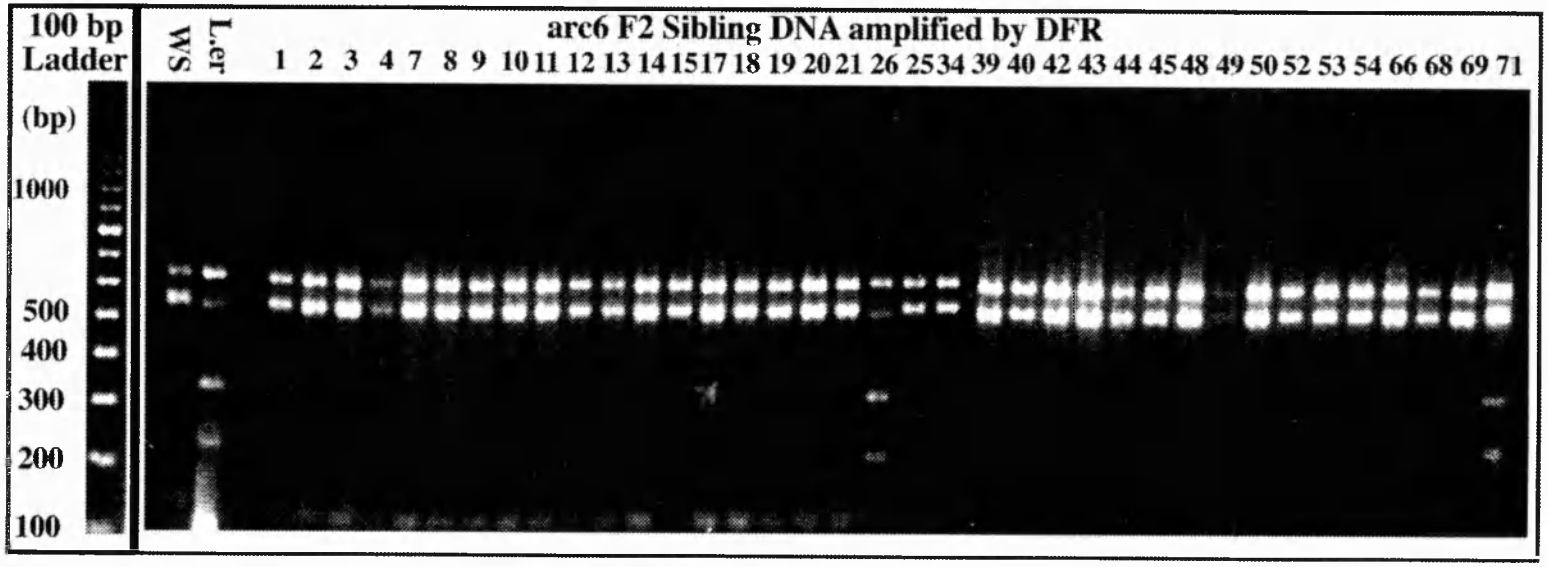
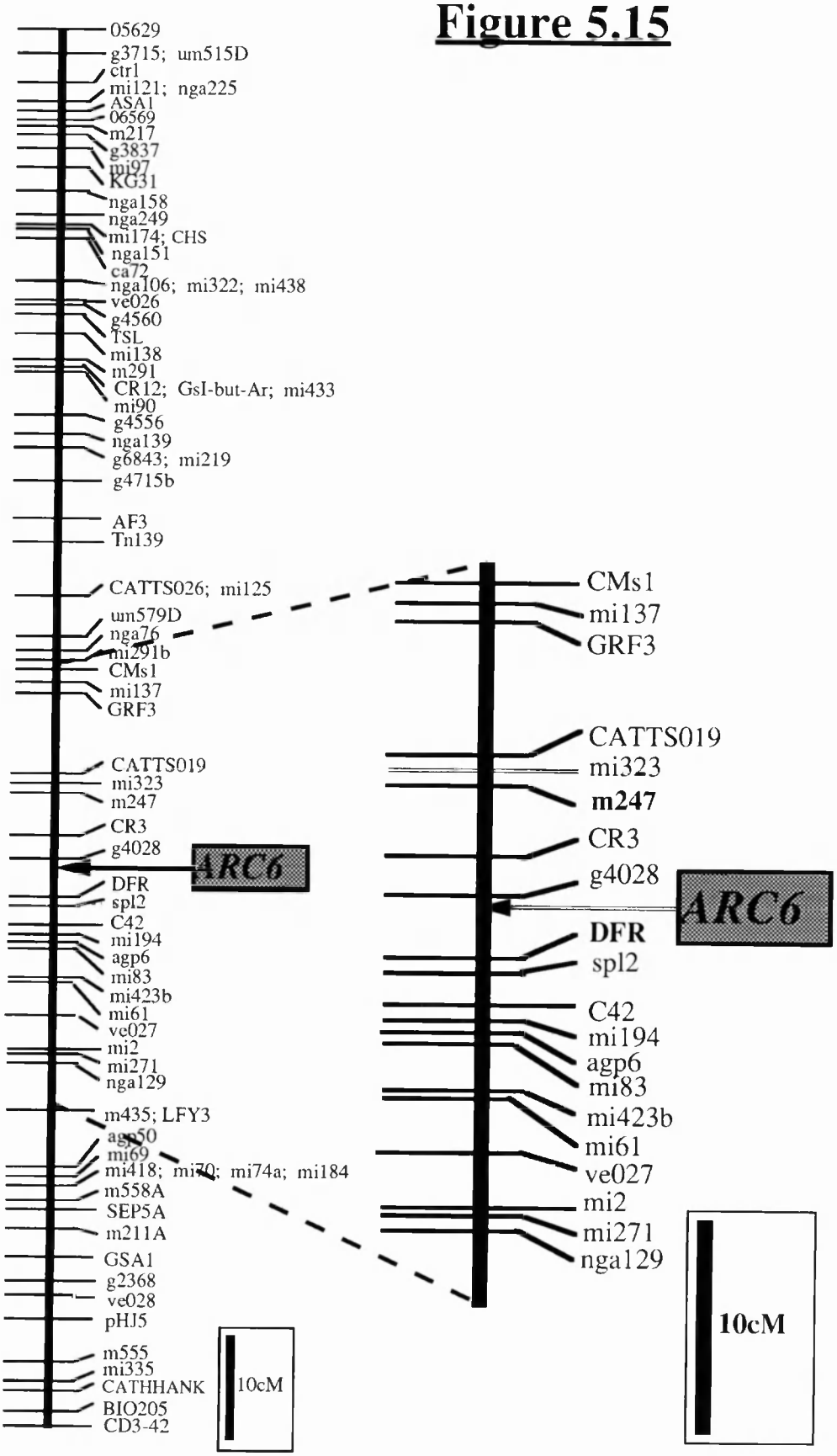

100 bp Ladder
(bp)
1000
500
400
300
200
100
arc6 F2 Sibling DNA amplified by DFR
WS
Ler
1 2 3 4 7 8 9 10 11 12 13 14 15 17 18 19 20 21 26 25 34 39 40 42 43 44 45 48 49 50 52 53 54 66 68 69 71

FIGURE 5.15 Proposed location of *ARC6* on chromosome 5

RFLP map of chromosome 5 of *Arabidopsis* indicating the proposed location of *ARC6*. The region between CMs1 and ngm129 in which *ARC6* is located is magnified.

Bar = 10cM.

Figure 5.15


05629
g3715; um515D
ctr1
mi121; nga225
ASA1
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mi97
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nga158
nga249
mi174; CHS
nga151
ca72
nga106; mi322; mi438
ve026
g4560
TSL
mi138
m291
CR12; GsI-but-Ar; mi433
mi90
g4556
nga139
g6843; mi219
g4715b
AF3
Tn139
CATTS026; mi125
um579D
nga76
mi291b
CMs1
mi137
GRF3
CATTS019
mi323
m247
CR3
g4028
ARC6
DFR
spl2
C42
mi194
agp6
mi83
mi423b
mi61
ve027
mi2
mi271
nga129
m435; LFY3
agp50
mi69
mi418; mi70; mi74a; mi184
m558A
SEP5A
m211A
GSA1
g2368
ve028
pHJ5
m555
mi335
CATHHANK
BIO205
CD3-42
10cM
CMs1
mi137
GRF3
CATTS019
mi323
m247
CR3
g4028
ARC6
DFR
spl2
C42
mi194
agp6
mi83
mi423b
mi61
ve027
mi2
mi271
nga129
10cM

The combination of the ARMS and CAPS mapping data has provided mapping cover across the majority of the *Arabidopsis* genome (figure 5.16). There is no cosegregation data for chromosome 3, but the very tight linkage of *ARC*6 to DFR and m247 is sufficiently close that the possibility of an error in the map location is very low. The lack of data showing that *ARC*6 does not map to chromosome 3 is not problematic.

The location of *ARC*6 on the *Arabidopsis* genetic map has proven fortuitous for the more rapid isolation of the gene than by conventional chromosome walking. The YAC contig. on chromosome 5 is well developed and is relatively close to completion. The availability of YAC clones which span the *ARC*6 region may prove of great value to the isolation of the gene. The location of *ARC*6 is also located within 15cM of the mapped *Ds* element *Ds*116, produced by Dr G. Coupland (unpublished). *Ds*116 may therefore be used in the future for directed mutagenesis with the *Ds* element to produce a tagged mutant allele of *arc*6.

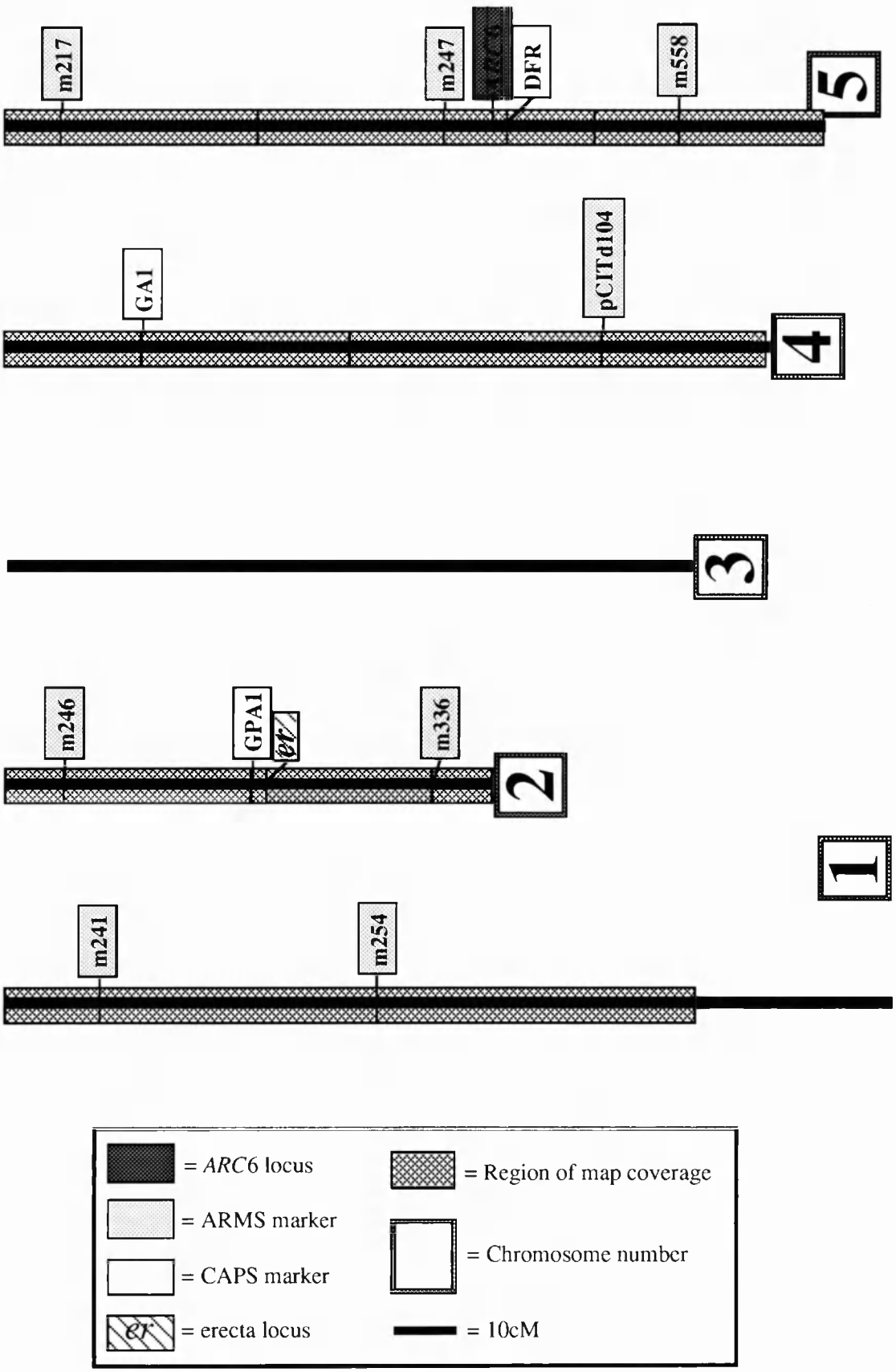
5.4 DISCUSSION

The extreme nature of the *arc*6 mutant phenotype stimulates several questions concerning the control of chloroplast division and the plasticity of the accumulation of the chloroplast complement in the mesophyll cells of higher plants. The low number and abnormally large size of *arc*6 plastids demonstrates the extremes to which the compensation of chloroplast size for chloroplast number may extend. The mesophyll cells of the *arc*6 mutant may have as few as a single chloroplast per leaf mesophyll cell, compared to the WS wild type mean chloroplast number of 80. The twisted leaf phenotype is the only permanent significant morphological effect of an *arc* mutation on leaf development. The distortion of mesophyll cell shape in the leaf is also unique to *arc*6 of all the *arc* mutants studied. The mutation also appears to affect germination and flowering time of the *arc*6 plant. Studies of the rate of biomass accumulation of *arc*6 seedlings (Pyke, Rutherford, Robertson and Leech, 1994) indicated that *arc*6 seedlings accumulate biomass in the early stages of development at a reduced rate compared to wild type.

FIGURE 5.16 Map coverage of the ARMS and CAPS mapping

RFLP map of *Arabidopsis thaliana* indicating the ARMS and CAPS markers used for the mapping of *arc*6. The region of map coverage of each of the markers is indicated by the hatched areas of the chromosomes. Coverage assumes a 50cM region of the chromosome covered by each marker. Bar = 10cM

Figure 5.16


m217
m247
ARC6
DFR
m558
5
GA1
pCITd104
4
3
m246
GPA1
er
m336
2
1
m241
m254
= ARC6 locus
= ARMS marker
= CAPS marker
er = erecta locus
= Region of map coverage
= Chromosome number
= 10cM

The existence of fertile, vigorous plants with such an extreme chloroplast mutant phenotype as *arc*6 questions the necessity for chloroplast division in *Arabidopsis* mesophyll cells. It is likely that the highly conserved phenotype of wild type chloroplasts is the result of refinement over the evolution of *Arabidopsis* mesophyll cells, producing the optimum chloroplast number and size. An analysis of *arc*6 and other *arc* mutant plants in conditions of stress would reveal the nature of the disadvantage of a non-wild type chloroplast complement. A preliminary study of the relative fitness of *arc*6 and WS wild type plants has been undertaken by M. Davies, an undergraduate student in our laboratory. Davies's results suggest that *arc*6 plants do not compete as well as wild type plants when the two genotypes are grown together. One may therefore predict that the *arc*6 mutation is deleterious (but not lethal) to the *Arabidopsis* plant when grown in competition. Since *Arabidopsis* is a ruderal species which may grow on low fertility soils the difference in fitness between *arc*6 and wild type is likely to have a major effect on the survival of the *Arabidopsis* plant when it germinates under these conditions.

The complex phenotype of *arc*6 therefore requires the consideration of the cause and effect of each *arc*6 phenotypic trait. The *arc*6 mutation may solely affect the division of plastids whose resultant phenotype affects cell shape and leaf shape. Alternatively, the mutant leaf and cell phenotype could affect the development of the plastid. The third possibility is that the *arc*6 mutation is pleiotropic and affects two developmental processes independently to produce the plastid and leaf mutant phenotypes. Further investigation of the *arc*6 chloroplast, cellular and leaf phenotypes is needed to establish which of these alternatives describes the endogenous leaf relationships.

The radical reduction in chloroplast number noted in *arc*6 cells of all types (Robertson, Pyke and Leech, 1995) suggests a strong lesion in the plastid division process. The chloroplast number in *arc*6 mesophyll cells very rarely increases beyond three chloroplasts and the lack of accumulation of chloroplast number with increasing cell size indicates that chloroplast division does not occur in *arc*6 mesophyll cells. This observation proposes the question of how plastid division is suppressed in the *arc*6 cell.

The extremely low chloroplast number of *arc*6 mesophyll cells indicates that the *arc*6 lesion affects an earlier process than chloroplast division, i.e. proplastid division. A lesion in proplastid division is shown by the low number and mutant morphology of the *arc*6 proplastid and its large size in the meristematic cell. The *arc*6 proplastids appear to be larger and more irregularly shaped than wild type, often being seen to wrap around the nucleus of the immature cell (Figure 5.5). The large size and broad, flat morphology of the *arc*6 proplastid is likely to cause a hindrance to proplastid division and may also cause a lesion in the later division of the chloroplasts which mature from the proplastids.

An absence of proplastid division in *arc*6 cells would rapidly cause a loss of proplastids from meristematic cell lines but no mature mesophyll cells which completely lack chloroplasts have been noted in *arc*6 leaves. *arc*6 proplastids must therefore be able to accumulate in number during cell division in the mutant. The control of the process of the partitioning of proplastids at cell division into an equivalent population in each daughter cell in *Arabidopsis*, or indeed any other species, is not fully understood. The proplastids of the lower vascular plant *Isoetes lacustris* segregate by the single, large proplastid, which is wrapped around the nucleus, being partitioned into the daughter cells after a period of active pleiomorphism of the plastid (Whatley, 1986). This means of proplastid segregation may possibly be similar in *arc*6 which is observed to have very few proplastids, which are of an irregular shape and which are wrapped around the pre-division nucleus. However the irregular shape of the *arc*6 proplastid does not necessarily indicate that the *arc*6 proplastid is sufficiently pleiomorphic to allow this type of division to occur. Several putative division profiles have been observed in *arc*6 proplastids (Robertson, Pyke and Leech, 1995) suggesting that proplastid division may occur at a reduced rate in *arc*6 compared to wild type. The majority of *arc*6 proplastids observed are larger than wild type, however, and these may be of a size or morphology incompatible with conventional proplastid division in *Arabidopsis*. The division of *arc*6 proplastids might therefore be limited to a small sub-population of proplastids which have not yet expanded beyond the morphological state compatible with division. An alternative possible explanation for the ability of *arc*6 proplastids to divide is that conventional proplastid division may be *completely inhibited* and division of the proplastid occurs by another method which is presently unknown.

The extremes to which chloroplast expansion may proceed is well illustrated by the *arc*6 mutant chloroplasts. The expansion of the *arc*6 chloroplasts to maintain a constant chloroplast complement in the mesophyll cell during cell expansion supports my suggestion that chloroplast expansion is a continuous process which may be interrupted periodically by chloroplast division (discussed in chapter 4). The complete lack of chloroplast division in *arc*6 mesophyll cells causes no apparent hindrance to chloroplast expansion suggesting that the two processes are largely independent. The expansion of the *arc*6 mutant chloroplast also illustrates the rigidity of the means of control which limits the accumulation of the chloroplast complement. The extensive size of the *arc*6 chloroplast is consistently constrained to a proportion of the mesophyll cell similar to that of wild type without the effect of plastid division as a modifying factor. This suggests that the limitation to the accumulation of the chloroplast complement is either a physical restraint by the expanding cell or a limitation to the resources for the development of the chloroplasts.

The evidence of abnormal proplastid development in the undifferentiated meristematic cells indicates that the *arc*6 mutant plastid phenotype is evident before the cellular expansion in the leaf primordium and the growing leaf. It is unlikely, therefore that the *arc*6 chloroplast phenotype is caused by the *arc*6 mutant leaf or cellular phenotype, although it is plausible that the reverse may be the case.

The twisted leaf effect of the *arc*6 mutation is observed in the primary leaves of *arc*6, associated with an alteration in mesophyll cell shape. The twisted leaf phenotype of *arc*6 may be caused either as a result of an altered mesophyll cell development which induces distortions in the cell shape and subsequently distortions in the leaf; or by an alternative process such as differential rate of expansion of the mesophyll cells to the epidermal cells of the leaf.

An alteration in the construction or polarity of the cellulose fibres in the cell wall could potentially alter the dimensions for expansion of the cell (Cleland, 1987) or may induce buckling of the expanding cell. The construction of the cell wall is heavily reliant on the action of microfilaments (Folson and Brown, 1987), which may also have a role in plastid division (Hashimoto, 1986) possibly indicating a pleiotropic effect of *ARC*6.

An alternative cause of the twisted leaf mutant phenotype may be the differential expansion of the mesophyll and epidermal cell layers in the expanding *arc*6 leaf. The differential rate of expansion of a layer of cells relative to adjacent layers induces buckling (Green 1985; Selker, Steucek and Green, 1992; Green, 1993). A disequilibrium in the development of the epidermis and mesophyll cell layers in the *arc*6 leaf as a result of differential cell expansion may be predicted to induce a similar buckling effect about the major vascular bundle of the leaf, the midvein. The *arc*6 leaf is observed to have a reduced cross sectional area (Pyke, Rutherford, Robertson and Leech, 1994) which suggests a reduction in the expansion of the leaf, possibly restricted by a reduced epidermal expansion since the epidermal cells appear to be smaller in the *arc*6 leaf than in wild type. The increased expansion of the mesophyll cell layer relative to the surrounding epidermal cells during leaf development would be likely to cause a compression of the mesophyll cells, possibly resulting in the distortion of mesophyll cell shape.

The analysis of the *arc*6 mutant phenotype is likely to be limited to speculation without a clear understanding of the structure and role of the *ARC*6 gene whose mutagenised form induces the severe effects of the *arc*6 mutation. The lack of a tagged *arc*6 mutant has required the initiation of a chromosome walking strategy to isolate the *ARC*6 locus, the initialisation of which has required the localisation of the *ARC*6 locus on the *Arabidopsis* genetic map.

The use of ARMS and CAPS mapping sets has localised the *ARC*6 locus to the bottom half of chromosome 5, between the markers m247 and DFR. The location of *arc*6 is fortuitous for a map-based cloning strategy due to the extensive work being undertaken in Britain as part of the *Arabidopsis* genome project to provide contiguous mapped YAC and cosmid clones covering the whole of chromosomes 4 and 5. At present, *arc*6 falls just outside of the YAC contig on chromosome 5 (R. Schmidt, personal communication), but the field of research is progressing rapidly and is likely to afford a YAC contig containing the *ARC*6 gene in the near future.

The ARMS and CAPS RFLP mapping techniques were of considerable use in the localisation of *ARC*6. The use of a mapping set to localise the *arc*6 mutation to one of the

five *Arabidopsis* chromosomes has greatly accelerated the mapping of *ARC*6. The ARMS and CAPS mapping sets provide an efficient and relatively unambiguous alternative to mapping using morphological markers. The use of marker lines such as W100 were anticipated to be overly labour intensive for use in our lab. The ARMS marker set provided a relatively simple means of localising the mutant locus to one of the chromosome arms with a small population of F_2 recombinant individuals; however the system was not ideal. The lack of data on WS/Ler polymorphisms for the ARMS markers reduced the efficiency of the procedure. The use of sets of multiple ARMS markers as suggested by Fabri and Schäffner (1994) proved to be difficult to score accurately, requiring the ARMS markers to be tested in smaller groups than the recommended size of the mapping sets. However, the ability to use the same Southern blot filter for all ARMS markers has greatly accelerated the mapping strategy compared to conventional RFLP mapping which would require several different Southern blot filters of genomic DNA digested with many different enzymes. The CAPS markers were efficient as an addition to the ARMS data, providing additional markers for mapping in the region suggested by the ARMS, however the low number of WS/Ler polymorphisms in this mapping set would have reduced the potential effectiveness for the initial localisation of the *ARC*6 locus.

The combined use of ARMS and CAPS markers in the mapping of *arc*6 has provided cover of the entire *Arabidopsis* genome to within 50cM with the exception of chromosome 3. The mapping data do not exhibit cosegregation of the *arc*6 mutant phenotype with any markers other than m247 and DFR, indicating that the *arc*6-1 mutation is not the result of a significant chromosome translocation, such as has been observed in other T-DNA induced mutants (Castle, Errampalli, Atherton *et al*, 1993). The precise cause of the *arc*6-1 mutation is so far unclear, since the T-DNA of *arc*6-1 does not map close to the mutant locus. The isolation and sequencing of *arc*6-1 by other means than using the T-DNA will provide insight into the cause of the mutation.

Although the *arc*6 mutation was mapped to reasonable accuracy with reasonable efficiency, none of the mapping approaches adopted was ideal for the isolation of the *ARC*6 locus. However the mapping techniques are anticipated to prove more efficient in the isolation of EMS-mutagenised *arc* loci of the Ler ecotype.

Future work

The isolation of the *ARC*6 gene may proceed by two approaches, the further use of gene tagging or the use of chromosome walking from the mapped *arc*6 mutant locus. The phenotype and map location of *ARC*6 are both fortuitous for further gene tagging experiments. The twisted leaf phenotype will facilitate the further analysis of T-DNA and transposon mutagenised populations at a greatly increased rate to that described in chapter 3. The isolation of several alleles of *arc*6 caused by independent insertion events will greatly increase the potential of isolating a tagged allele of the mutant. An alternative means of tagging the *ARC*6 locus is provided by its fortuitous map position on chromosome 5, since the *ARC*6 locus lies within 8cM of a mapped inserted *Ds* element, *Ds*116. The initiation of transposition of *Ds*116 may be achieved by crossing the *Ds* line to a line bearing an *Ac* transposase source. The reinsertion of the *Ds*116 preferentially in the local region to the original *Ds* locus (Bancroft and Dean, 1993; Feldmann, Malmberg and Dean, 1994) is anticipated to provide a novel allele of *arc*6 tagged with *Ds*116.

The identification of the general location of *ARC*6 will facilitate the initiation of a chromosome walk to the locus. This long-term strategy first requires the fine mapping of *ARC*6 by a combination of RFLP mapping using a greater number of F_2 backcrossed progeny and mapping using physical markers to the *ARC*6 region of chromosome 5. The chromosome walking strategy will be greatly aided by the development of contiguous regions of YAC and cosmid clones from chromosome 5 (Schmidt, Cnops, Bancroft and Dean, 1992; R. Schmidt, personal communication).

The isolation of further tagged alleles of *arc*6 by more intensive screening and localised *Ds* transposition is likely to prove the most effective method for isolating the *ARC*6 locus. The chromosome walking strategy is a more long-term and labour intensive approach for gene isolation in *Arabidopsis* and may not be the most efficient method for cloning the *ARC*6 gene.

5.4 SUMMARY

The *arc*6 mutant, isolated from a T-DNA mutagenised population of *Arabidopsis* has a mesophyll cell phenotype with a mean of only two chloroplasts per cell. The chloroplasts of *arc*6 are up to 50 fold larger than wild type in plan area, but are functional and produce starch. The proplastids of the meristematic cells of the root and shoot apices are also large, indicating that *arc*6 is a mutation which also affects proplastid division.

The *arc*6 mutant is recognisable by its twisted leaves. The primary leaves of *arc*6 are buckled about the primary leaf axis and contain misshapen mesophyll cells. The altered chloroplast phenotype is seen in the meristematic cells and the leaf primordia prior to the development of the mutant leaf phenotype, suggesting that the leaf mutant phenotype is not the cause of the chloroplast mutation.

Neither of the two alleles of *arc*6 isolated are tagged with a functional T-DNA which would have been of use in the isolation of the *ARC*6 locus. An alternative map-based cloning strategy was initiated using the ARMS and CAPS mapping sets modified for use with WS ecotype to localise the *arc*6 mutation to the lower half of chromosome five between the markers m247 and DFR. The identified locus of *ARC*6 is situated near to YAC contigs and a mapped *Ds* element, *Ds*116, which are anticipated to be of significant use in the subsequent isolation of the *ARC*6 gene.

CHAPTER 6

The Isolation of DNA Sequences from the *ARC*11 Gene

6.1 INTRODUCTION

6.1.1 The characterisation of transposable elements in plants

The ability of the transposon to excise from a genetic locus and reinsert at another site has made the technique of transposon-mutagenesis in plants potentially a very powerful tool for the isolation of novel plant mutant phenotypes. The approach has also been adopted in recent years for the isolation of genes which are tagged by the mutagenic presence of the transposon at the gene locus (reviewed in Bhatt and Dean (1992) and Gierl and Saedler (1992)). This approach of transposon tagging was adopted for the generation of *arc* mutants in *Arabidopsis* as an alternative means of gene tagging from the use of T-DNA insertional mutagenesis which has so far proven to be ineffective for producing a tagged *arc* allele. One mutant, *arc*11, was isolated from transposon-mutagenised populations of *Arabidopsis thaliana*. *arc*11 displays a mutant phenotype of highly variable chloroplast number and size compared to wild type. The isolation of the *ARC*11 gene using transposon tagging is the central aim of this chapter.

The existence of transposable elements in plants was first confirmed by Barbara McClintock in the middle of this century. Evidence of the spontaneous and variable mutation of several loci in maize (*Zea mays*) prompted the investigation of a genetic factor which stimulated localised mutation in a large number of the progeny of a single plant (McClintock, 1952). The co-ordination of this effect with mitosis was also observed, suggesting that the unknown genetic factor controlled the timing or frequency of mutation and was active in cells in which the chromatids had undergone the 'breakage-bridge-fusion' cycle in early anaphase (McClintock, 1952). This variable mutable phenotype had also been observed previously by Emerson concerning the P locus (Rhoades, 1992), an effect which was later proven to be caused by the *Ac* element.

The identification of a locus which cosegregated with this mutability facilitated the further analysis of the phenomenon; the locus was termed *Dissociation* (*Ds*) by McClintock . The mutation frequency, caused by the presence and action of *Ds* suggested an identity for the mutable genetic factor. Subsequent analysis revealed a second genetic locus, also located on chromosome 9, whose presence was required for the activity of *Ds*.

This locus, termed *Activator* (*Ac*) is now understood to code for a nuclear DNA-binding protein, 'Transposase' (Fedoroff, 1992) which facilitates the excision and reinsertion of either the *Ac* or *Ds* elements. *Ac* is therefore an autonomous element which may excise by itself; *Ds*, however, requires the presence of *Ac* for excision to occur.

The analysis of the cosegregation of *Ds* with local genetic markers *C, sh, bz and wx* illustrated a variable genetic location for the *Ds* element, indicating in 1948 that the *Ds* was mobile; similar results were observed later with *Ac* (Fedoroff, 1992). The existence of mobile genetic elements was treated with suspicion by the majority of the scientific community until the discovery of 'Insertion sequences' in *E. coli* (Shapiro, 1969; Starlinger and Saedler, 1972) and P elements in *Drosophila* (Green, 1992). Other transposable elements have been subsequently isolated in maize, such as the *Enhancer-Inhibitor/Suppressor-mutator* (*En/Spm*) system characterised separately by both Peterson and McClintock (Saedler and Starlinger, 1992)) and the 'Mutator' (*Mu*) system (Robertson, 1985). Transposable elements have also been identified in *Antirrhinum* (*Tam* elements), and *Arabidopsis* (Voytas and Ausubel, 1987; Peleman, Cottyn, Van Camp, Van Montagu and Inzé, 1991; Tsay, Frank, Page, Dean and Crawford, 1993).

The precise mechanism of *Ac/Ds* transposition has not been fully explained to date. The *Ac* element is typically 4563 bp in length and codes for a single mRNA containing four introns and a long, untranslated leader sequence of no known function (Fedoroff, 1983; Kunze, Stochaj, Laufs and Starlinger, 1987). The transcription product, '*Transposase*', of the *Ac* element is an 807 amino acid polypeptide whose function is to bind to DNA where it recognises an AAACGG hexamer (Kunze, Stochaj, Laufs and Starlinger, 1987). This hexamer is present in multiple copies at both ends of the *Ac*, and presumably has some function in the reinsertion of the *Ac* into the genome since deletions in the AAACGG regions reduces or abolishes transposition. The protein involved in chromosome breakage observed by McClintock (1952) to accompany *Ds* insertion into the chromatid is not known. The means by which transposition of the *Ds* is activated by the *Ac* is also not fully understood at present.

McClintock's early work observed the activity of transposable elements after mitotic or meiotic activity of the cell. Transposition frequency is relatively rare, less than

one excision per generation (Saedler and Starlinger, 1992). The mechanism of the control of transposition is presently also a subject of speculation. McClintock observed that the excision frequency of *Ac* was inversely proportional to the *Ac* copy number in the genome. It is likely that this effect is caused by the production of transposase, since the copy number of *Ds* (which is often observed to be similar in sequence to *Ac*) does not exhibit an effect on *Ac* or *Ds* excision frequency (Bancroft and Dean, 1993*a* and 1993*b*).

6.1.2 Insertional mutagenesis by transposable elements

Gene isolation by the tagging of loci with an inserted DNA fragment was first achieved in bacteria by Kleckner, Chan, Tye and Bostein (1975) and in plants by the tagging of the *bronze* mutation of maize by *Ac* (Fedoroff, Furtek and Nelson, 1984). Since these early approaches to gene tagging, many loci have been isolated by gene tagging with a number of endogenous and heterologous transposable element systems in a variety of species (reviewed by Bhatt and Dean, 1992; Coupland, 1992; Gierl and Saedler, 1992; Walbot, 1992; and Feldmann, Malmberg and Dean, 1994)). The use of the maize transposable elements *Ac/Ds*, *En/Spm*, and *Mu* has provided the widest range of isolated mutants. The use of maize and *Antirrhinum* transposable elements in gene tagging of heterologous species has recently also been pioneered in tobacco, tomato, rice and *Arabidopsis* (for a review of heterologous tagging systems, see Bhatt and Dean (1992)). The activity of *Ac* in particular has been most closely studied in several species such as tobacco (Baker, Schell, Lörz and Fedoroff, 1986), *Arabidopsis* and carrot (Van Sluys, Tempé and Fedoroff, 1987) Tomato (Yoder, Palys, Alpert and Lassner, 1988) and Rice (Murai, Li, Kawahoe and Hayashimoto, 1991); more recently efficient tagging systems involving *Ac/Ds* have been pioneered in tomato (English, Harrison and Jones, 1993) and *Arabidopsis* (Dean, Sjodin, Lawson, *et al* , 1990; Bancroft, Bhatt, Sjodin *et al*, 1992). No endogenous transposons in these species have yet been sufficiently developed for use as a gene tagging system.

The advantages of transposons are considerable for gene tagging. The autonomous transposable elements such as *Ac* may transpose independently during plant development, thus potentially generating limitless new mutant individuals. Alternatively, the non-

autonomous elements, such as *Ds* may be distributed to regions across the genome where they will be sufficiently stable to allow the development of homozygous lines bearing a single transposon at a defined locus (Bancroft and Dean, 1992). Such lines of plants bearing mapped *Ds* elements will facilitate localised transposition when provided with an *Ac* transposase source. The potential for reversion of the transposon-induced phenotype is of considerable value to gene tagging; the mobile transposon may re-transpose in a mutant individual by excision of the element from the mutant locus. The excision of the transposon will frequently restore the function of the gene, enabling a **revertant** individual to be observed which displays a wild type phenotype in the progeny of a mutant plant. Reversion of the mutant phenotype to wild type is very strong evidence that the mutant was tagged by a functional transposon.

6.1.3 Transposon tagging in Arabidopsis

The genome of *Arabidopsis* has been shown to contain several endogenous transposable elements, however these elements are not yet sufficiently characterised to be employed as an effective gene tagging system. The gene fragments isolated from the study of the *Tal*-10 (Voytas and Ausubel, 1988) and the *Tat*1 alleles (Peleman, Cottyn, Van Camp, Van Montagu and Inzé, 1991) revealed motifs and repeat sequences reminiscent of heterologous transposable elements; however these elements are not transposed during normal development or during gametogenesis. Recent work by Tsay, Frank, Page, Dean and Crawford (1993) on the isolation of the *CHL*1 gene has revealed the presence of another transposable element in *Arabidopsis*, the *Tag*1 element. The analysis of *Ac* mutagenised populations of Landsberg *erecta* seedlings revealed a *chl*1 mutant which contained a 3.3kb insert in the *CHL*1 gene which did not hybridise to a radio-labelled *Ac* clone. Sequence analysis of this fragment revealed that it was not *Ac* but did retain certain characteristics of a transposon. Subsequent analysis of the *Tag*1-mutagenised *chl*1 allele revealed that *Tag*1 could excise from the gene locus, restoring the wild type phenotype. Analysis of wild type genomic DNA revealed the presence of *Tag*1 in the genome of Landsberg *erecta* ecotype but not WS or Columbia ecotypes. The *Tag*1 element is the first

mobile endogenous transposable element to be isolated in *Arabidopsis* and represents a significant advance for gene tagging in this model genetic system

The Enhancer-Inhibitor (*En-I*) transposon system of maize has recently been adopted for the successful transposon tagging and isolation of the male sterility gene *MS2* in *Arabidopsis* (Aarts, Dirkse, Stiekema and Pereira, 1993). The *En-I* sequences were used in the confirmed mutant to amplify plant flanking DNA which was itself used to probe a cDNA library to isolate clones of the *MS2* gene.

The principal investigation of the activity of transposable elements in *Arabidopsis* has concentrated on the *Ac/Ds* system (Coupland, 1992; Bancroft, Bhatt, Sjodin, *et al*, 1992). The activity of *Ac* in tobacco and tomato was observed to be high, compared to maize. The activity of *Ac* in *Arabidopsis*, however, was observed to be up to 100 fold lower in *Arabidopsis* than in maize, which was too low to be useful for gene tagging (Schmidt and Willmitzer, 1989; Dean, Sjodin, Page, Jones and Lister, 1992). The construction of an increased activity *Ac* element by the deletion of a 537bp fragment from between positions 428 and 964 of the 5' untranslated leader sequence by *Nae*I digestion resulted in increased excision activity of the *Ac* in *Arabidopsis* (Lawson, Scofield, Sjodin, Jones and Dean, 1994). The reason why the *Nae*I deletion (Δ*Nae*I) increases the activity of *Ac* in *Arabidopsis* is unknown, since the deletion has a converse effect of reducing the activity of the element in tobacco (Bhatt and Dean, 1992).

The development of a two element tagging system using *Ac/Ds* in *Arabidopsis* is described in detail by Bancroft, Bhatt, Sjodin *et al* (1992). The deletion of the 3' terminus of the *Ac* inhibits the transposon's ability to excise without reducing its production of transposase. This stable *Ac* (*sAc*) may be used as a stable high-efficiency transposase source for the activation of *Ds* elements which require a transposase source to be mobile. The mutagenesis of *arc*11 was performed by the insertion of a mobile Δ*Nae*I *Ac* in a single-element tagging system.

The *Nae*I deleted mobile *Ac* (Δ*Nae*I *Ac*) was cloned into the antibiotic resistance gene streptomycin phosphotransferase (SPT) of the recombinant NPTII fusions (kanamycin resistant plasmid vectors), 02213 and 0383. The Δ*Nae*I *Ac* in the 02213 was

cloned in 5' to 3' orientation; the Δ*Nae*I *Ac* in 0383 was cloned in the 3' to 5' orientation. The *arc*11 gene was isolated from an 02213 transformant.

The analysis of the transposition frequency of these elements in *Ac* was facilitated by the use of a streptomycin assay (Dean, Sjodin, Page, Jones and Lister, 1992). Excision of the Δ*Nae*I *Ac* from the SPT gene enabled the reactivation of the gene, restoring streptomycin resistance to a plant in which excision had occurred. Streptomycin resistance was characterised by the appearance of green cells in the cotyledons of seedlings grown in the presence of streptomycin; streptomycin sensitive plants were fully bleached but could still germinate. Excision of the *Ac* in the somatic cell lineage of the germinating seedling resulted in a patchy, '**variegated green**' (**VG**) phenotype which showed isolated sectors of green tissue (in which *Ac* excision had occurred) within a bleached cotyledon (in which *Ac* excision had not occurred) depending on when excision had occurred in the cell lineage. A '**fully green**' (**FG**) phenotype in which the whole seedling was green when grown on streptomycin, demonstrated that excision had occurred in the germline of the parent plant of that seedling. Only fully green phenotypes were of use in gene tagging, since the excision event in a fully green plant had occurred in the germline and therefore *all* the cells of the seedling contained the same *Ac* excision and reinsertion event.

The aim of the work described in this chapter was to identify individuals which were mutant for chloroplast division and were transposon tagged. The ultimate aim being to use such tagged individuals to clone and sequence *Arabidopsis* chloroplast division genes. A screen of the Δ*Nae*I *Ac*-mutagenised populations revealed a single mutant phenotype, *arc*11. Genetic analysis of *arc*11 showed that *arc*11 is a recessive mutation of a nuclear *ARC* gene which is not allelic to existing *arc* mutant alleles. *arc*11 mesophyll cell chloroplasts are very variable in number and size between individual cells, however the *arc*11 whole plant phenotype is not significantly different to wild type.

The analysis of the transposon mutagenised populations has provided the first tagged *arc* mutant of *Arabidopsis* and facilitated the isolation of the first *ARC* gene in higher plants. The characterisation of the *arc*11 mutant and *ARC*11 gene sequence is anticipated to aid the study of chloroplast division and expansion in higher plants.

6.2 RESULTS

6.2.1 The arc*11 mutant*

Seed from FG seedlings of individual transgenic plants transformed with either the 02213 and 0383 recombinant T-DNA were screened for evidence of a mutant chloroplast phenotype in the mesophyll cells. The FG phenotype of the parent plant indicated that excision of the *Ac* from the 02213 T-DNA had already occurred, so that mutant phenotypes isolated were likely to be the result of insertional inactivation by the Δ*Nae*I *Ac* rather than the T-DNA.

The *arc*11 mutant was isolated from the progeny of FG #122 from the 02213 transformed population '02213-3' of the Δ*Nae*I *Ac* mutagenised lines. Twelve individual seedlings were screened from line #122; two displayed an *arc*11 mutant chloroplast phenotype; two were heterozygous for the *arc*11 mutation and eight were homozygous wild type. The mutant chloroplast phenotype of the *arc*11 mutant was analysed and its tagged nature verified by the analysis of cosegregation of *arc*11 with the *Ac* and by reversion of the chloroplast phenotype to wild type by the excision of the *Ac* element.

The *arc*11 chloroplast phenotype is one of the most variable *arc* phenotypes noted to date. A typical *arc*11 cell, with a wide range of chloroplast sizes is shown in figure 6.1 in comparison to wild type. The *arc*11 cell has a variable chloroplast number, about a mean of 33 chloroplasts per mesophyll cell (wild type mean of 121). These chloroplasts range in size from half the size of wild type to *c*.10 fold larger than wild type. The *arc*11 mutant cells show a wide and apparently random distribution in chloroplast sizes and numbers which is similar to the chloroplast phenotype of *arc*2, rather than the phenotype of two distinct sizes of chloroplasts within the cell as is noted. The *arc*11 whole plant phenotype is not significantly dissimilar to that of wild type, and the growth and vigour of the *arc*11 plant is normal under growth room conditions. The backcrossing of *arc*11 to Columbia wild type demonstrates the *arc*11 mutation to be a nuclear recessive mutation which exhibits normal Mendelian inheritance characteristics. Reciprocal allelic crossing between *arc*11 and other *arc* mutants has demonstrated that the *arc*11 mutant represents an independent novel *ARC* locus in *Arabidopsis*.

Figure 6.1

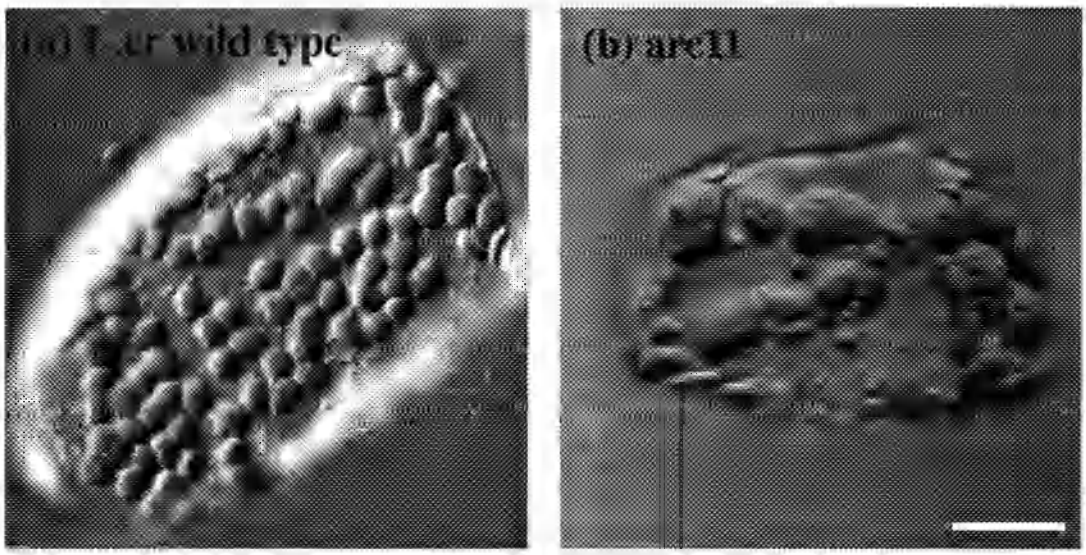


FIGURE 6.1 **Ler wild type and *arc*11 mesophyll cells**

Isolated mesophyll cells of *(a)* wild type (Landsberg *erecta*) and *(b) arc*11 mutant.

Bar = 25µm

6.2.2 The analysis of the Ac*-tagged status of* arc*11*

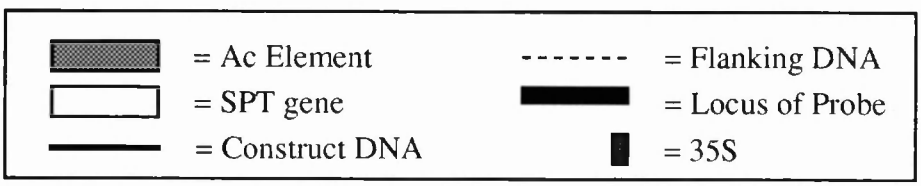
The investigation of the tagged status of *arc*11 was approached by two methods; ***(a)*** The analysis of the cosegregation of the *arc*11 mutation with the *Ac* element in the progeny of a backcross to wild type; and ***(b)*** The investigation of the progeny of a single mutant plant for somatic and germinal revertants.

(a) The cosegregation of the Ac **element with the** arc*11 mutation*

The presence of a functional *Ac* element which cosegregated fully with the *arc*11 mutation in the F_2 progeny of a backcross to wild type would indicate that the *arc*11 mutant was tagged by the insertion of the *Ac*. 44 mutant F_1 siblings from the progeny of one of the two original heterozygote plants isolated from the 02213-3 #122 family; and 22 F_2 mutant siblings from a backcross of *arc*11 to Columbia wild type were analysed for cosegregation of the *arc*11 mutation with the presence of *Ac*. The lack of a selectable marker, such as kanamycin resistance (as was used in the T-DNA mutants), on the Δ*Nae*I *Ac* element required the use of Southern blotting to reveal the presence of the *Ac* by the use of a DNA probe which recognised a fraction of the *Ac* sequence (Dean, Sjodin, Page, Jones and Lister, 1992). Figure 6.2 shows the configuration of the 02213 T-DNA and the Δ*Nae*I *Ac* both before and after excision of the *Ac* from the T-DNA. The location of the *Nae*I 537 bp deletion is indicated, near to the 5' end of the *Ac*. The location of *Ssp*I restriction sites are also indicated in figure 6.2. The site of recognition of the 0.9kb *Ac* probe is indicated in figure 6.2; the probe spans a region containing a close cluster of *Ssp*I sites. Digestion with *Ssp*I will therefore produce two fragments which are recognised by the *Ac* probe; a **0.93kb** fragment of *Ac* DNA and a second fragment containing both the *Ac* DNA as well as foreign DNA since there is no *Ssp*I site towards the 3' end of the *Ac* sequence. This second fragment recognised by the *Ac* probe will be either **2.55kb** in an *Ac* which is still resident in the T-DNA; or **1.5kb or more**, depending on the location of the next *Ssp*I site in the adjacent plant tissue in an *Ac* which has transposed to a new locus. The Δ*Nae*I *Ac* is resident in the SPT gene of the 02213 T-DNA before excision, inactivating the gene. Upon excision, the SPT gene is re-annealed and reactivated. The 400bp fragment of the SPT gene, used as a probe to the SPT gene (Dean, Sjodin, Page,

FIGURE 6.2 Map of the Δ*Nae*I element

Diagram of the Δ*Nae*I *Ac* element resident within the 02213 T-DNA. *(a)* The Δ*Nae*I *Ac* element when resident within the donor site of the 02213 T-DNA SPT gene. The recognition sites of the enzyme *Ssp*I are indicated. The regions of hybridisation of the *Ac* and SPT DNA probes are also indicated, with the predicted DNA fragment sizes which would be recognised by each probe in an *Ssp*I digest. The site of the 537bp *Nae*I deletion is shown, proximal to the 5' end of the Δ*Nae*I *Ac* . *(b)* The Δ*Nae*I Ac element and the 02213 T-DNA after excision from the SPT donor site. Altered fragment sizes for the recognition of *Ssp*I digested DNA by the *Ac* and SPT probes are shown. Bar = 500bp.



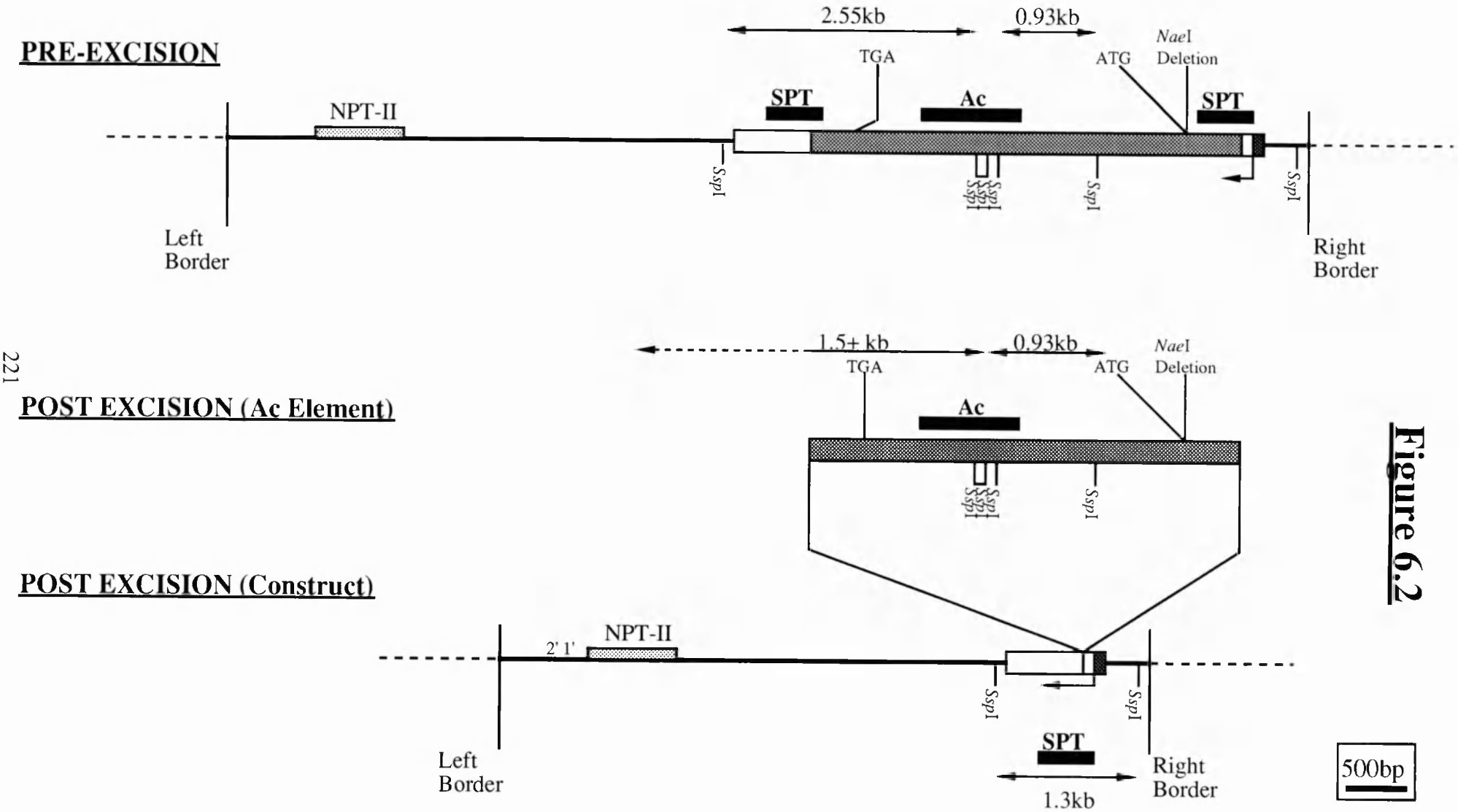

PRE-EXCISION
2.55kb
0.93kb
TGA
ATG
NaeI
Deletion
SPT
Ac
SPT
NPT-II
SspI
SspI
SspI
SspI
SspI
Left
Border
Right
Border
POST EXCISION (Ac Element)
1.5+ kb
0.93kb
TGA
ATG
NaeI
Deletion
Ac
SspI
SspI
SspI
SspI
POST EXCISION (Construct)
2' 1'
NPT-II
SspI
SspI
SPT
1.3kb
Left
Border
Right
Border
500bp

Figure 6.2

Jones and Lister, 1992) to verify the lack of a resident *Ac* in the T-DNA of the *arc*11 mutant, is shown in figure 6.2. The SPT probe recognises SPT sequences which span the *Ac* donor site. When digested by *Ssp*I, the DNA of an 02213 T-DNA with a **resident *Ac*** will reveal the 2.55kb fragment (also recognised by the *Ac probe*) and 2.1kb fragment when probed with the SPT probe (figure 6.2). The *empty donor site* of the SPT gene in a T-DNA with an **excised *Ac*** will recognise a single fragment in an *Ssp*I digest, of **1.3kb**.

The results of the Southern blot hybridisations of the 0.9kb *Ac* probe to the genomic DNA of 66 mutant sibling individuals are illustrated in figure 6.3(*a*) to (*c*). The wild type DNA shows no hybridisation to the *Ac* probe; however the mutant parent and 52 of the mutant siblings display the predicted **0.93**kb fragment as well as a **2.5**kb fragment, indicating that the *Ac* element was present in these mutant sibling individuals. The DNA of 7 of the mutant siblings tested was not cut by the *Ssp*I restriction enzyme, however the uncut genomic DNA of these individuals, which is present near the top of the Southern blot filter, *did* hybridise to the *Ac* probe in all 7 samples, suggesting that *Ac* was also present in these individuals. 59 of the 66 mutant sibling individuals therefore showed evidence of the presence of the *Ac* in their genomic DNA. However, **7** of the 66 siblings tested showed **no evidence** of an *Ac* element when probed with the *Ac* probe. The lack of complete segregation between the *arc*11 mutation and the *Ac* suggests that a functional Δ*Nae*I *Ac* element is not present in the DNA of all of the mutant siblings. The lack of an *Ac* in some mutant individuals may be due to one of three reasons: ***(i)*** Recombination has occurred between *Ac* and *arc*11 mutant loci which are closely linked but not at the same position; ***(ii)*** The *Ac* element present in these mutants is not detectable by the 0.9kb *Ac* probe; or ***(iii)*** The mutants have been caused by an *Ac* element which has subsequently excised from the *ARC*11 locus, but has left a mutagenic footprint in the gene, causing the *arc*11 mutant phenotype to be retained. The determination of the cause of the lack of cosegregation between *Ac* and *arc*11 requires the amplification by PCR and sequence analysis of the plant gene sequences which flank the locus of *Ac* insertion. The precise locus of the *Ac* insert would be revealed by the amplification of this DNA region from *Ac*-containing mutants. Evidence of a deletion at the region of *Ac* insertion in the

FIGURE 6.3 The cosegregation of *arc*11 with *Ac*

Autoradiographs of Southern blots of mutant siblings from the *(a), (b)* F_1 generation of an individual which was heterozygous for *arc*11 and *(c)* F_2 generation of a backcross between *arc*11 and Columbia wild type. 22 mutant siblings are included on each Southern blot with *arc*11 parental and wild type DNA. The DNA was digested with *Ssp*I restriction enzyme and the southern blots probed with the 0.9kb *Ac* probe. Two bands of 0.9 and 2.5 kb each are revealed in 52 of the 66 individuals. 7 individuals did not cut with *Ssp*I but do hybridise to the *Ac* probe, indicated by white arrows. 7 individuals do not hybridise to the *Ac* probe, indicated by black arrows. Size markers from a *Hin*dIII/*Eco*RI-digested λ DNA ladder are shown in kb.

Figure 6.3 (a)

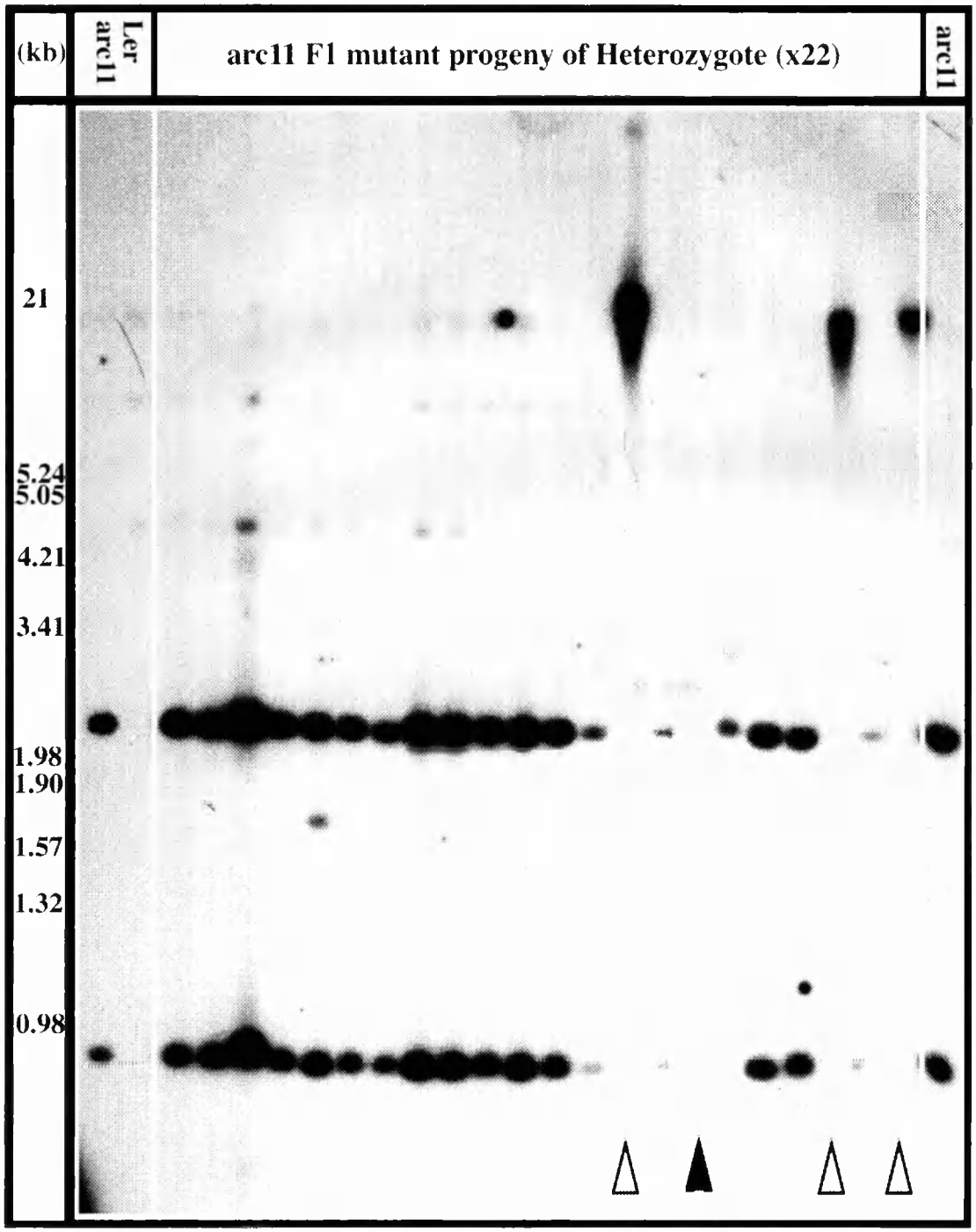

(kb)
Ler arc11
arc11 F1 mutant progeny of Heterozygote (x22)
arc11
21
5.24
5.05
4.21
3.41
1.98
1.90
1.57
1.32
0.98

Figure 6.3 (b)

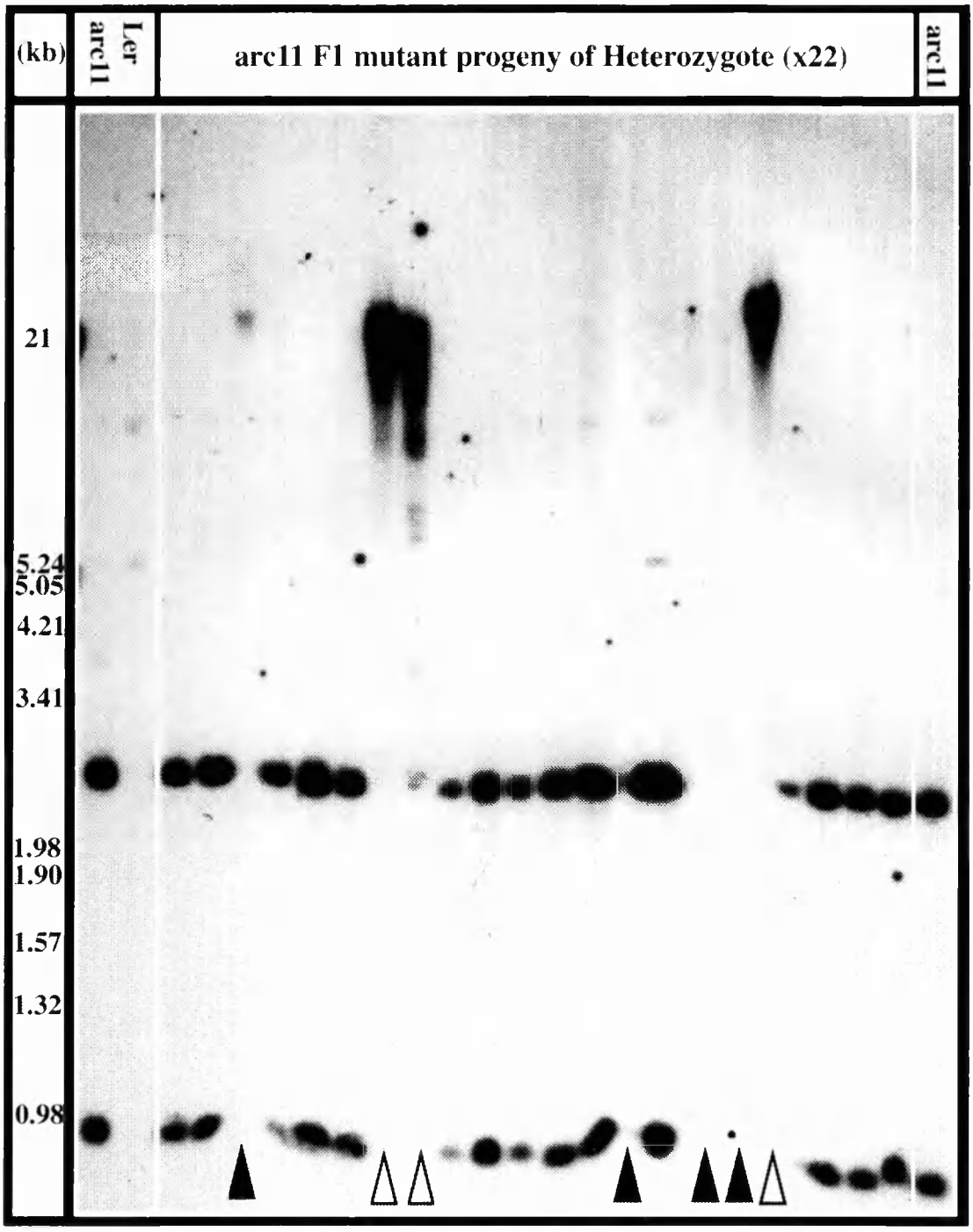
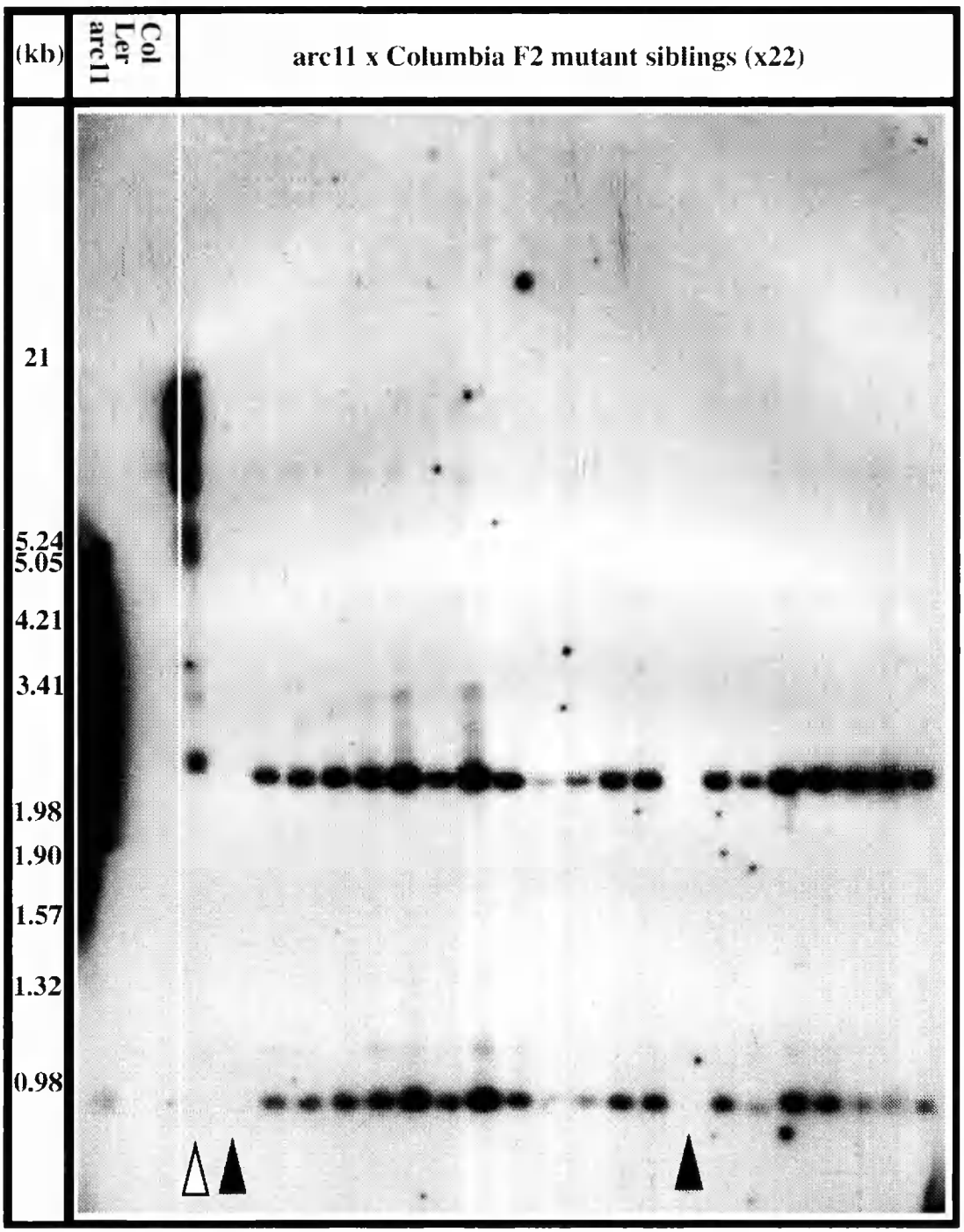

(kb)
Ler arc11
arc11 F1 mutant progeny of Heterozygote (x22)
arc11
21
5.24
5.05
4.21
3.41
1.98
1.90
1.57
1.32
0.98

Figure 6.3 (c)


(kb)
Col
Ler
arc11
arc11 x Columbia F2 mutant siblings (x22)
21
5.24
5.05
4.21
3.41
1.98
1.90
1.57
1.32
0.98

amplified gene sequence of the non-*Ac* containing siblings when compared to that of a wild type parent would indicate that a mutagenic footprint was present and therefore that the locus had once contained an *Ac*. A lack of a recognisable footprint in the DNA at this locus in non-*Ac* bearing mutant plants would indicate that this locus of *Ac* insertion was not the *ARC*11 locus and that the presence of *Ac* was only coincidental to the mutation of the *arc*11 gene and not its cause.

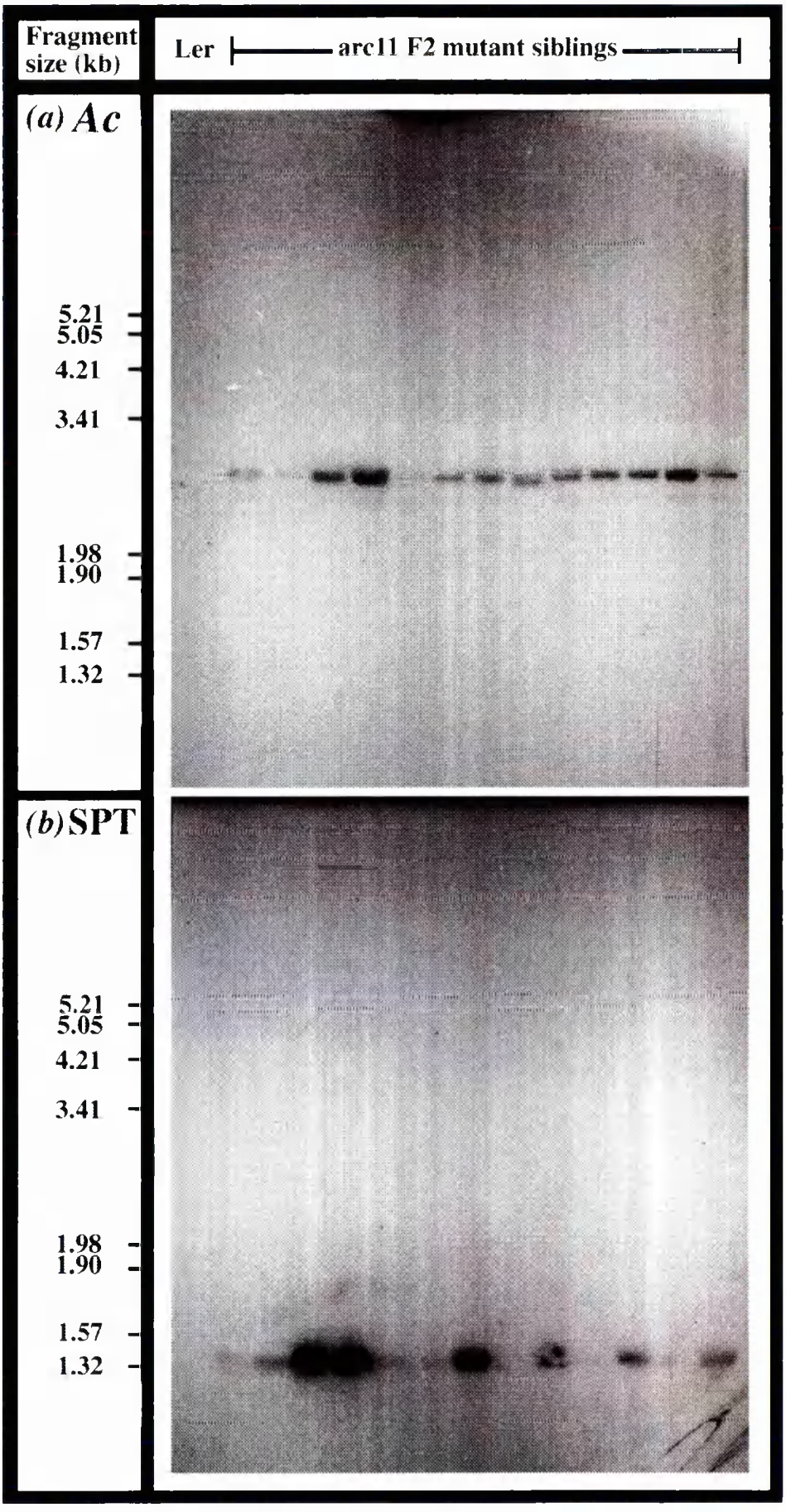
The 2.5kb band observed in the Southern blots of figure 6.3 (*a, b, and c*) is of a similar size to the 2.55kb band which would have been predicted from an unexcised *Ac* which was resident within the 02213 T-DNA. To verify that the 2.5kb fragment represented the DNA from an excised *Ac* element the *Ssp*I digested *arc*11 genomic DNA was probed with a fragment of the SPT gene. The SPT DNA probe was expected to recognise a 1.3kb fragment in *Ssp*I-digested genomic DNA if the SPT gene of the T-DNA contained an *empty donor site*, i.e. there was no *Ac* present. The autoradiographs of a single Southern blot of 12 *Ssp*I-digested *Ac* sibling DNAs, probed successively with the *Ac* and then with the SPT probes are illustrated in figure 6.4. The 2.5kb band observed in figure 6.3 was revealed by the *Ac* probe, as would have been expected. A single 1.3kb fragment was observed in the mutant DNAs when probed with SPT, indicating that the SPT gene in the 02213 T-DNA contained an empty donor site from which an *Ac* had excised. The 2.5kb fragment was therefore representative of a transposed *Ac*.

The cosegregation of the *arc*11 mutation with the SPT locus was also investigated to verify that the *arc*11 mutant was not caused by the 02213-3 T-DNA insertional event. 44 of 66 individual siblings tested showed cosegregation with the 02213-3 T-DNA. Since the T-DNA cannot normally excise from its genetic locus in the plant (Martineau, Voelker and Sanders, 1993), the lack of complete cosegregation of the *arc*11 mutation to the T-DNA indicates that the *arc*11 mutant is not caused by the T-DNA. The high degree of cosegregation does, however, suggest that the *arc*11 locus is located very close to the 02213-3 T-DNA locus which has been mapped by C. Lister (personal communication) to the top of chromosome 5. A map position of *arc*11 which is close to the 02213-3 T-DNA

FIGURE 6.4 *arc*11 DNA probed with *Ac* and SPT probes

Autoradiographs of a single *Ssp*I-digested Southern blot of genomic DNA from 12 mutant F2 siblings from a backcross of *arc*11 to wild type probed with *(a) Ac* and *(b)* SPT DNA probes. The *Ac* probe reveals a 2.5kb band, similar in size to that observed in figure 6.3; the SPT probe reveals a 1.3kb band, indicative of an empty donor site within the SPT gene, caused by transposition of the *Ac*. Size markers from a *Hin*dIII/*Eco*RI-digested λ DNA ladder are shown in kb.

Figure 6.4


Fragment size (kb)
Ler
arc11 F2 mutant siblings
(a) Ac
5.21
5.05
4.21
3.41
1.98
1.90
1.57
1.32
(b) SPT
5.21
5.05
4.21
3.41
1.98
1.90
1.57
1.32

supports the findings of Dean and co-workers that *Ac* and *Ds* transposition occurs primarily in the region of the original location of the transposon element (Coupland, 1992; Bancroft and Dean, 1993*a*; Bancroft, Jones and Dean, 1993; Feldmann, Malmberg and Dean, 1994).

(b) The analysis of arc*11 plants for evidence of reversion to wild type*

The reversion of the progeny of an *arc*11 mutant plant to a wild type chloroplast phenotype would indicate the presence of an active *Ac* element at the *ARC*11 locus. Germinal reversion, where the *Ac* element had excised in the germline, would be characterised by a wild type chloroplast phenotype in 100% of the mesophyll cells in an offspring plant of a mutant parent. Somatic reversion, where the *Ac* had excised from the somatic cells during mitosis in the shoot apex would be characterised by sectors of mesophyll cells which display a wild type chloroplast phenotype beside *arc*11 mutant cells in a single leaf. The size of somatic revertant sectors would be dependent on the timing of the excision event in the dividing meristematic cell lineages of the apex. The effect of reversion of the *arc*11 phenotype to wild type would be strong evidence that the *arc*11 mutant was induced by a functional *Ac* element.

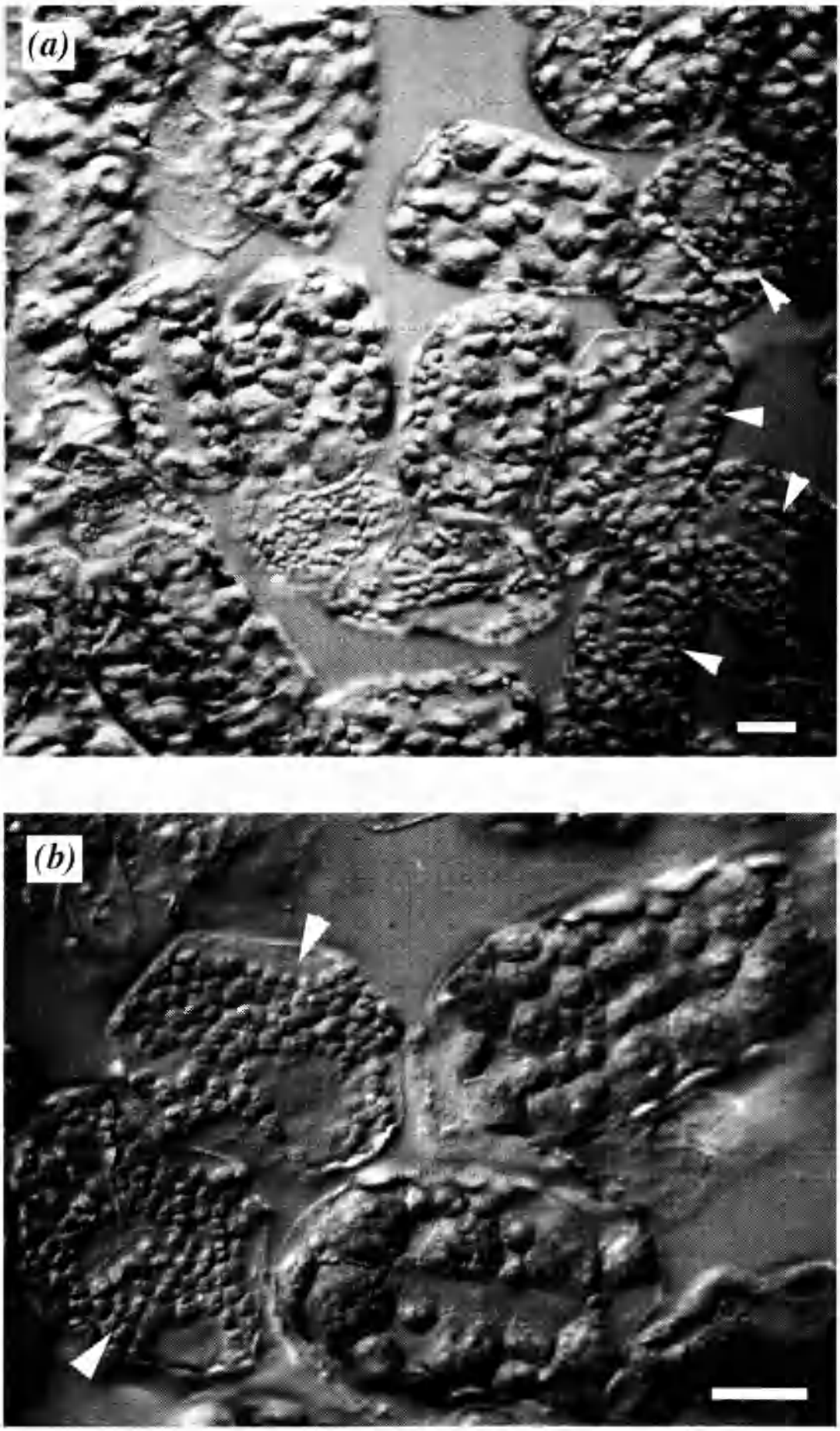
The whole of a first leaf from each of 1200 individual mutant siblings germinated from the seed of a single *arc*11 mutant plant was screened microscopically by eye for evidence of reversion of the mutant chloroplast phenotype to a wild type phenotype in the mesophyll cells. The leaves were fixed and digested in EDTA as described in 2.3.1; the EDTA-treated leaf was placed on a microscope slide and gently pressed under a coverslip. The mesophyll cells of the leaf were separated sufficiently to be distinguishable under the microscope without the structural integrity of the leaf being too distorted. Sectors of mesophyll cells displaying a wild type phenotype beside cells which were mutant were resolvable by this technique.

18 individual seedlings demonstrated evidence of sectors of mesophyll cells which showed a wild type chloroplast phenotype which were distributed within regions of cells displaying an *arc*11 mutant chloroplast phenotype within a single leaf (illustrated in figure 6.5). These sectors are evidence of somatic reversion at a low (1.8%) frequency in the

FIGURE 6.5 Somatic revertant mesophyll cells of *arc*11

Photomicrographs of mesophyll cell squashes of the first leaf of an *arc*11 seedling demonstrating somatic reversion of the mesophyll cell mutant phenotype to that of wild type. Wild type cells, indicated by arrows, are observed towards the right hand side of figure 6.5*(a)* and the left hand side of 6.5*(b)*. Bar = 25μm

Figure 6.5



development of the first leaf. This frequency was lower than would be expected from *ΔNae*I *Ac* (Dean, Sjodin, Page, Jones and Lister), but this may be due to the sub-cellular screening procedure not resolving all revertant individuals.

No seedlings were isolated from the screen of the 1200 offspring of the *arc*11 mutant plant which displayed a fully wild type mesophyll cell phenotype in the first leaves. The lack of any wild type seedlings indicated an absence of germinal reversion in the 1200 seedlings screened. However the analysis of progeny from each of the 18 somatic revertant plants showed one of these plants to segregate 1:1 for wild type and mutant progeny. The existence of wild type progeny from a mutant plant which had displayed revertant sectors in its first leaves was evidence of a germinal reversion. The segregation of the progeny of the revertant plant indicated that an excision event had occurred early in one germline of the somatic revertant parent individual, effectively causing the plant to become heterozygous in one germline, thus producing equal numbers of wild type and mutant progeny.

The existence of both somatic and germinal revertants in the progeny of a mutant *arc*11 plant was taken as strong evidence that the *arc*11 mutant ***is tagged*** with a functional *Ac* element which may excise to restore a wild type phenotype. The revertant evidence was taken as sufficient proof of the tagged status of *arc*11 for the isolation of the *arc*11 gene to proceed. However, the analysis of the *ARC*11 DNA sequence of the non-*Ac* bearing mutant siblings for mutagenic *Ac* excision footprints, is still required for complete confidence that the *arc*11 mutant is tagged.

6.2.3 The amplification of the ARC*11 plant DNA sequences flanking the* Ac *element by IPCR*

The DNA of *arc*11 mutant, *arc*11 F_2 mutant siblings and wild type was digested with *Bst*YI and *Bcl*I restriction enzymes and subjected to IPCR as described in section 2.9.2. The genomic DNA was cut with both enzymes in order to minimise the size of the plant flanking DNA fragments so that IPCR could proceed more efficiently on smaller fragments. Two independent IPCR experiments were carried out using primers to the 3'

and 5' ends of the Δ*Nae*I *Ac* respectively. The primers DL6 and D71 were used for the 3' region, and the primers B34 and D74 were used for the 5' region of the *Ac*. The genomic restriction fragments were circularised and the *Ac* flanking sequences were amplified by PCR. The 3' IPCR reaction produced two fragments of 0.8kb and 1.1kb respectively; the 5' IPCR reaction produced a single 0.2kb fragment (illustrated in figure 6.6).

The three IPCR fragments were isolated and cloned in the *Eco*RV site of the M13-based plasmid pKR (J. Jones, personal communication). Since the IPCR fragments were amplified from the *Ac*, each fragment should contain *Ac* DNA sequences, and would therefore recognise similar *Ac* sequences in the genomic DNA of an *Ac*-containing *arc*11 mutant. The IPCR fragments should also recognise *ARC*11 gene sequences in both *arc*11 and wild type genomic DNA. The insertion of the *Ac* into the *ARC*11 locus of the mutant plants will cause any restriction fragments which span this region to be of different size in mutant than in wild type. The digestion of mutant and wild type DNA with *Ssp*I, which cuts internally to the *Ac* will produce a fragment in the mutant which will be recognised by the 3' IPCR fragment as the same 2.5kb restriction fragment recognised by the *Ac* probe. This is because the *Ssp*I enzyme should cut at the same site in the plant DNA as was described in 6.3.2. The 5' fragment will recognise a c.1.3+kb fragment since the *Ssp*I will cut at the site near to the 5' end as well as an unknown site within the plant flanking DNA. The use of either probe in wild type should light up a similar sized fragment equivalent in size to the 3' and 5' recognised fragments but without the 2.8kb of *Ac* sequences (1.5kb + 1.3kb) which are present in the mutant. The digestion of the wild type and mutant the DNA by an enzyme which cuts externally to the *Ac*, such as *Eco*RV, will reveal the same fragment when probed with either the 3' or 5' fragments. However, this fragment will be larger in mutant DNA compared to wild type DNA due to the presence of the 3.3kb *Ac* insertion within the DNA. The verification that the IPCR fragments hybridise to DNA restriction fragments of different size in wild type and *arc*11 mutant would indicate that the cloned fragments were amplified from the *ARC*11 DNA at the site of the *Ac*.

Figure 6.6

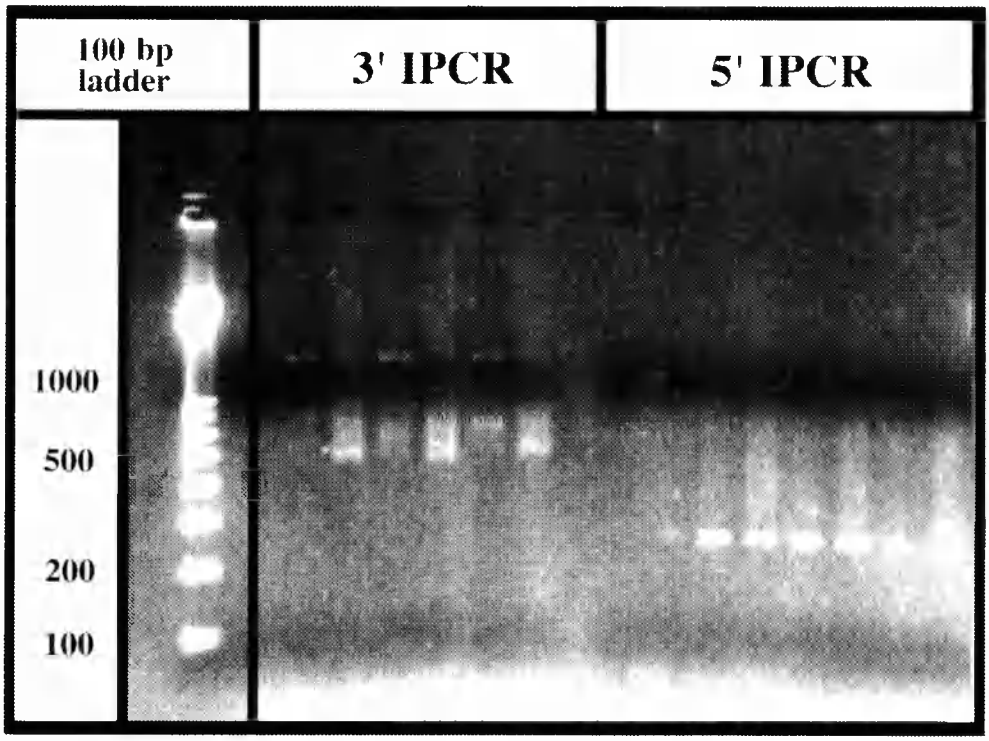


FIGURE 6.6 DNA fragments isolated by IPCR of arc11 locus

Photograph of a 2% agarose gel containing aliquots of the resultant DNA from the 3' and 5' IPCR experiments to amplify the genomic DNA flanking the *Ac* sequence. The 3' IPCR samples contain two bands, of 1.1kb and 0.8kb size. The 5' IPCR samples contain a single 0.2kb band.

The results of hybridisations of the 1.1kb 3' and the 5' fragment to *Ssp*I and *Eco*RV digested mutant and wild type parental and sibling DNAs are illustrated in figure 6.7*(a)* and *(b)*. The 1.1kb 3' fragment (figure 6.7*(a)*) did not hybridise to the plant DNA in the *Ssp*I digested material. Since the *Ssp*I and *Eco*RV digested individuals are from identical DNA samples between digests, any sequence which is recognised in the *Eco*RV DNA should also be recognised in *Ssp*I-digested DNA. The lack of hybridisation to the *Ssp*I digested DNA indicates that the 6.5, 3.4 and 3.0kb bands observed are the result of the recognition of a contaminant from the *Eco*RV enzyme. This supposition is supported by the appearance of the same sized bands in the 5' probed Southern blot. The lack of the expected size difference between the DNA bands of wild type and mutant DNA when probed with the 3' IPCR fragment also supports the suggestion that the 1.1kb 3' fragment is not representative of the *ARC*11 gene. The lack of hybridisation of the 1.1kb 3' fragment to plant DNA suggests that the fragment does not contain plant DNA sequences and is rather a contaminant DNA sequence which was amplified by the IPCR. However, *Ac* sequences were observed in the DNA sequence of this fragment (see below), which indicates that the fragments *was* amplified by IPCR of an *Ac*.

The 5' IPCR fragment displays a band shift in fragment sizes between both *Ssp*I and *Eco*RV-digested DNA. The 5' probe recognised a 2.0kb band in wild type and a 2.2 kb band in the *arc*11 mutant DNA cut with *Ssp*I. The 5' probe also recognised a 5kb band in wild type and a c.8kb band in mutant DNA cut with *Eco*RV. The band shift in the *Ssp*I-digested DNA is 0.2kb in size; the band shift in the *Eco*RV-digested DNA is *c*.3kb in size, as was predicted. The 5' fragment is therefore almost certainly representative of the plant DNA flanking the *Ac* locus in *arc*11, and may be used in the analysis of the *ARC*11 locus

6.2.4 The sequence analysis of the IPCR fragments

The nucleotide sequences of the plant DNA flanking the *Ac* element for the first 349bp of the 1.1kb 3' fragment and the entire 5' fragment were determined by C. Lister and are illustrated in figure 6.8 *(a)* and *(b)* respectively. Both of the nucleotide sequences of the 1.1kb 3' fragment and the 5' fragment contained recognisable *Ac* sequence data at either end of the IPCR fragment.

FIGURE 6.7 *arc*11 DNA probed with the 3' and 5' IPCR products

Autoradiographs of two Southern blots of wild type and *arc*11 mutant DNA cut with *Ssp*I and *Eco*RV restriction enzymes which cut internally and externally to the *Ac*. Southern blots were probed with *(a)* The 1.1kb 3' IPCR fragment; *(b)* The 0.2kb 5' IPCR fragment. A difference in the size of bands is observed in the DNA of wild type compared to mutant when probed with the 5' IPCR fragment. No difference in band size is observed when probed with the 1.1kb 3' IPCR fragment. Size markers from a *Hin*dIII/*Eco*RI-digested λ DNA ladder are shown in kb.

Figure 6.7 (a)

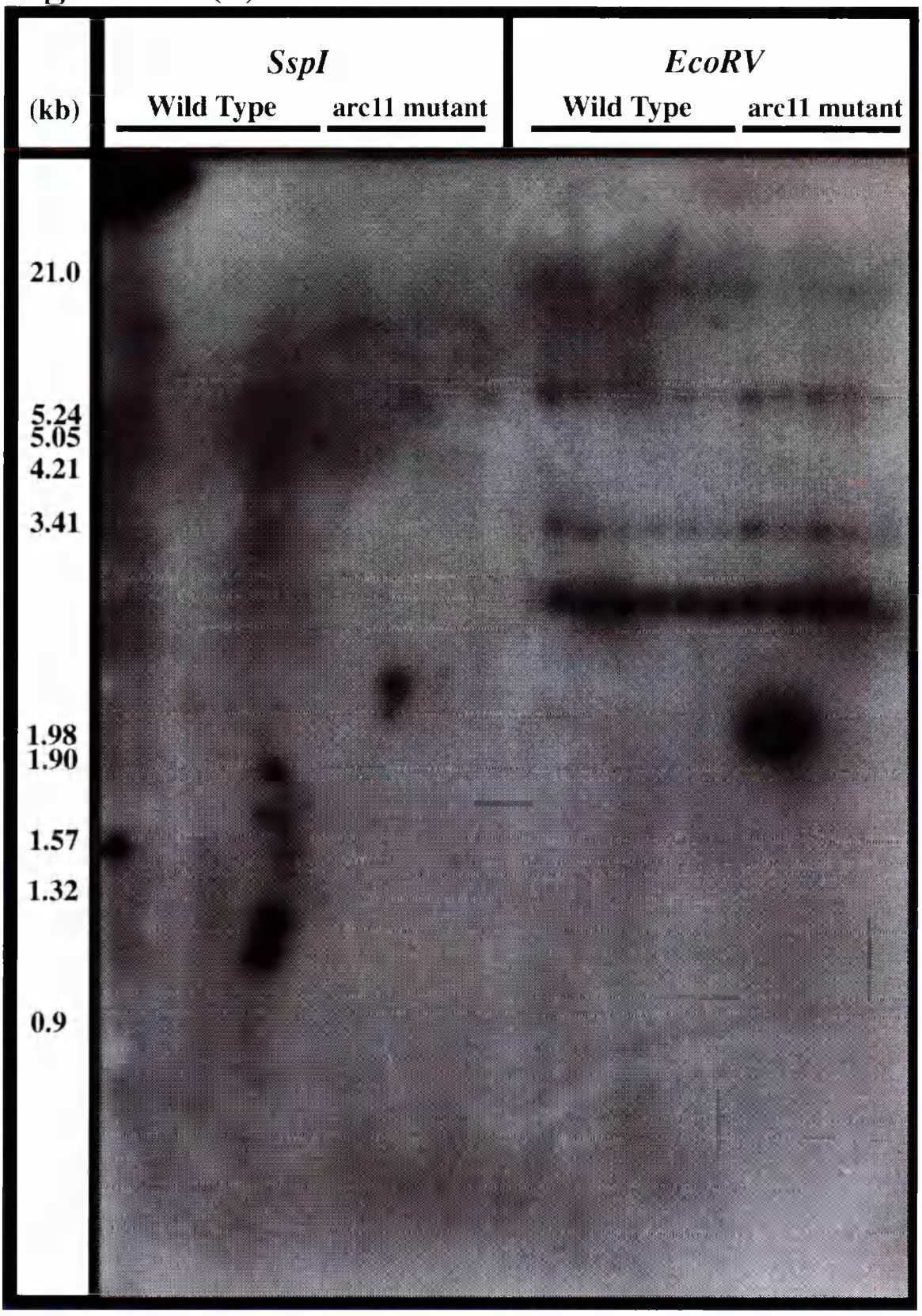

(kb)
SspI
Wild Type
arc11 mutant
EcoRV
Wild Type
arc11 mutant
21.0
5.24
5.05
4.21
3.41
1.98
1.90
1.57
1.32
0.9

Figure 6.7 (b)

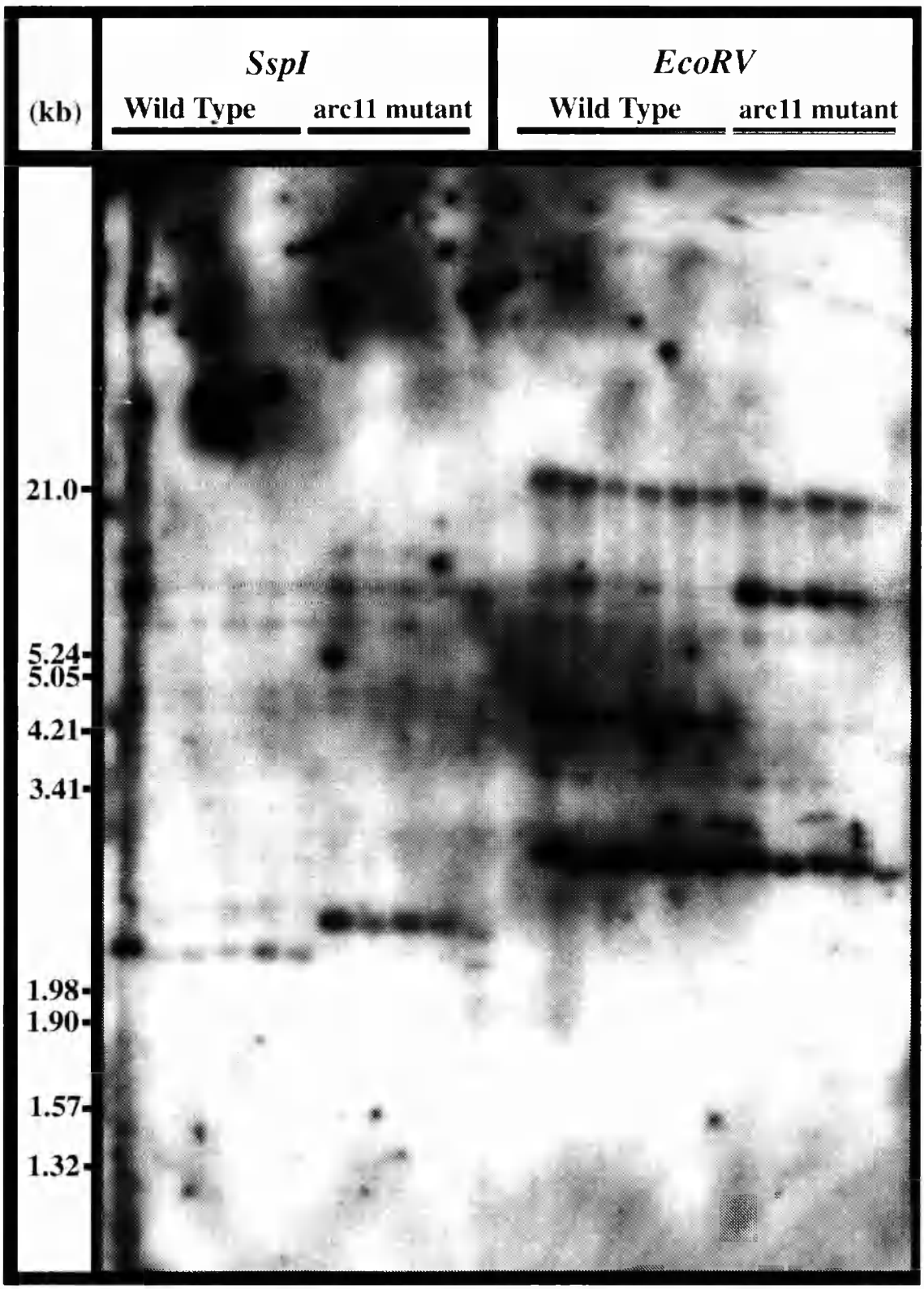

(kb)
SspI
Wild Type
arc11 mutant
EcoRV
Wild Type
arc11 mutant
21.0
5.24
5.05
4.21
3.41
1.98
1.90
1.57
1.32

Figure 6.8*(a)*

Nucleotide sequence of 349bp of the 1.1kb 3' IPCR fragment

TCCAG	ATCCA	ATACC	GCACT	AAGAA	TCGCC
1		11		21	
GGGTT	GTTGT	TCATA	GTCAG	AACAA	TGATC
31		41		51	
GACAG	GCTTG	GGAAA	TGGCG	CTTGA	TGTAC
61		71		81	
TTGAT	TAAGG	TAATG	CCATC	GCCGT	ACTTA
91		101		111	
TCGCC	AGGCA	TGGAG	AGATC	GGTAA	TCAAC
121		131		141	
ACATG	CGCAT	CCAGT	TTCGG	CAGGT	TGTTG
151		161		171	
ATCAG	TGCTG	TAGAG	TCTTC	AAATT	CGCCG
181		191		201	
ACAAC	ATTCA	CCCAC	TCAAT	TTGCT	CAAGT
211		221		231	
ATTTG	CGAAT	ACCGA	ACAAG	ACTAT	CGGAT
241		251		261	
GGGTC	ATCGG	CAATA	ATTAC	GTTCA	TATTG
271		281		291	
TTCAT	GTAAT	AGGCT	ACCTT	GCTAC	AGCAA
301		311		321	
GCCTC	TTGAC	ATAAC	TGTC		
331		341			

FIGURE 6.8 Nucleotide sequences of 3' and 5' IPCR fragments

Nucleotide sequence of the *(a)* first 349bp of the 1.1kb 3' IPCR fragment; and *(b)* The 150bp of the 5' IPCR fragment.

Figure 6.8*(b)*

Nucleotide sequence of the plant sequences of the 0.2kb 5' IPCR fragment

CTTGG	AAGCT	TCTCT	CTATC	ATATC	TACTC
1		11		21	
TTAAC	TGTTT	TTGCA	AOGGC	AGAAA	AACCA
31		41		51	
ACAGA	CTTGTT	AAAAT	CATCA	TGACA	CAGCC
61		71		81	
AAGTT	AAAGA	TCAAC	TAAAA	GACGT	AACTG
91		101		111	
ACTTT	GTGTG	AAGAG	TAGTG	CAGAT	C
121		131		141	

An amino acid sequence from both IPCR nucleotide sequences was predicted in each of three frames in both the forward and reverse orientations, since no start codons were discernible in the nucleotide sequences. Homology searches for both the nucleotide sequences and all six predicted amino acid sequences were performed for both IPCR fragments using the GENBANK computer database. No significant nucleotide sequence homology was observed with the 5' IPCR fragment. In the case of the 1.1kb 3' fragment, significant (99.8%) homology was observed to the *rcsB* and *rcsC* capsule synthesis genes of *E. coli* (Jayaratne, Keenleyside, MacLachlan, Dodgson and Whitfield, 1993). The 349bp sequence of the 1.1kb 3' fragment recognised a 349bp overlap of sequences from the *rcsB* gene between positions 4030 4377 in the identical *rcsB and rcsC* gene sequences. The very high (99.8%) homology of the 1.1kb 3' fragment to an *E.coli* gene is further evidence in addition to the hybridisation results shown in figure 6.7(*a*) which suggests that the 1.1kb fragment may not have originated from the *ARC*11 locus since such high homology would not be expected between a plant and a bacterial gene.

No significant homology was found to any of the predicted amino acid sequences for either the 1.1kb 3' or the 5' IPCR fragment.

6.3 DISCUSSION

The mutant chloroplast phenotype of *arc*11 displays considerable variety both between different mesophyll cells and within the single cell. The determination of chloroplast number and size in the *arc*11 mesophyll cell is apparently random, producing a wide range of chloroplast number, size and shape so no predictable phenotype for the number and size of the chloroplasts in the chloroplast complement may be made. This mutant phenotype has been noted previously to a less extreme extent in the *arc*2 mutant which also displays a variable chloroplast phenotype per mesophyll cell. The radical changes in the chloroplast population of the *arc*11 mesophyll cell do not alter the total chloroplast area of the cell in comparison to wild type and have no visible effect on the vigour or fertility of the *arc*11 plant as a whole.

The *arc*11 mutation is not as extreme a phenotype as observed in several other *arc* mutants; however, the variable phenotype is of considerable interest in the analysis of the co-ordination of division within the chloroplast population of the mesophyll cell. The apparently random distribution of the chloroplast numbers and sizes in the *arc*11 mesophyll cells suggests that the process of chloroplast division in this mutant is not completely perturbed but rather is retarded sufficiently so that it becomes unpredictable whether division will proceed. The range of chloroplast size implies that chloroplast division has occurred in some *arc*11 chloroplasts but not in others. The larger chloroplasts have probably undergone very few, if any, chloroplast divisions in the development of the mesophyll cell, whereas the smaller chloroplasts are likely to have arisen from a lineage of chloroplasts in which division has regularly occurred. The cause of a differential rate in chloroplast division is unclear, however the lack of increased numbers of chloroplast division profiles, as is observed in *arc*5 mesophyll cells, suggests that the *arc*11 lesion affects the initiation of chloroplast division rather than its completion.

The lack of any other significant whole plant mutant phenotype in *arc*11 suggests that the *arc*11 mutation exclusively affects the chloroplast division process rather than being pleiotropic. The *arc*11 mutant is therefore of considerable interest to the study of the regulation of the initiation of chloroplast division in *Arabidopsis*. The isolation of the *ARC*11 gene is anticipated to be of considerable importance in the study of chloroplast division in higher plants.

The tagged nature of the *arc*11 mutant is still not fully confirmed, although the observation of somatic and germinal reversion is sufficient evidence to allow the isolation of the *ARC*11 gene to proceed with relative certainty. However the cause of mutant individuals which do not bear the *Ac* element needs to be investigated for complete assurance that the *arc*11 mutant is caused by the insertion of a Δ*Nae*I *Ac* element.

The lack of cosegregation of the Δ*Nae*I *Ac* with the *arc*11 mutation may be due to one of two events.

(i) The *ARC*11 gene may have been mutagenised by another means independent of the Δ*Nae*I *Ac* so that the *arc*11 mutation is closely linked to the Δ*Nae*I *Ac* locus, but not

caused by the insertion of a transposon. The cosegregation data presented in figure 6.3 would suggest an approximate genetic distance of 10.6cM between the *ΔNaeI Ac* and the *arc*11 locus. An alternative cause of the mutagenesis of the *ARC*11 locus would therefore be implied if the *ΔNaeI Ac* was not the mutagen. One possibility is that the *arc*11 mutation is caused by the insertion of an additional, truncated *Ac* element, independent of the *Ac* element detected in the Southern blots of figure 6.3, and not recognised by the 0.9kb *Ac* probe. The endogenous *Tag*1 transposable element present in Landsberg *erecta* (Tsay, Frank, Page, Dean and Crawford, 1993) is not detectable by probes to *Ac*; it is possible that the *arc*11 mutation was induced by *Tag*1 at a site which was c.10cM from the *Ac* locus. Alternatively, the *arc*11 mutation may be due to the somaclonal variation induced by the tissue culture techniques by which the T-DNA was introduced to the plant material (Feldmann and Marks, 1986). The transformed populations were screened for visible mutations before the excision of the *Ac*, but this screen did not include an analysis of the cellular phenotype (Dean, Sjodin, Lawson *et al*, 1990). Another possible cause of the *arc*11 mutation could be that the mutagenesis was by the 02213 T-DNA insert rather than the transposon insertional event. This suggestion could be tested by examining all of the 02213-3 mutant lines. If the *arc*11 mutation had arisen from the insertion of the T-DNA, progeny of the 02213-3 transformant, which all contain the T-DNA at the same locus, would show an *arc*11 phenotype. Since only one FG family of 02213-3 showed an *arc*11 phenotype, the T-DNA is not the mutagen of *arc*11. Furthermore, the lack of complete cosegregation of the *arc*11 mutation with the SPT gene resident in the T-DNA indicates that the T-DNA is not resident at the *arc*11 locus.

(ii) The alternative explanation for the mutant individuals which do not contain *Ac* is that these individuals have lost the transposon through excision of the element, but retain a mutant phenotype due to a mutagenic footprint after the excision of the *Ac*. The evidence of somatic revertant sectors within a single plant and germinal reversion progeny of mutant individuals strongly suggests that the mutation is caused by an inserted *Ac* element which can excise, reverting the chloroplast phenotype of the cell to wild type. It is therefore unlikely that the *arc*11 mutation was caused by an alternative means to the *Ac*. It is most likely that the individuals which are mutant for *arc*11 but which lack an *Ac* are

individuals in which the *Ac* element has excised from the *ARC*11 gene leaving a mutagenic footprint. The sequences of several excision footprints involving nucleotide deletions have been determined by Bancroft, Jones and Dean (1993). Footprints which cause a frame shift in the revertant gene sequence by the loss of a complete codon after excision of the transposon usually retain the wild type phenotype. However, footprints in which the deleted nucleotides cause an incomplete frame shift of less than a codon usually result in such a frame shift retaining the mutation of the gene sequence. The presence of a mutant footprint would cause a mutant phenotype in the cell without the presence of an *Ac* element in the mutant gene.

In the case of *arc*11, the footprint explanation needs further investigation. The comparison of the *ARC*11 gene sequences of the individuals which are deficient for an *Ac* to those of wild type and *Ac*-containing mutants will indicate the presence or absence of a footprint. The sequence data from the 5' IPCR fragment may then be used to synthesise PCR primers to the *ARC*11 gene. *ARC*11 PCR primers will enable the region of DNA which spans the *Ac* insertion to be amplified and sequenced. DNA amplified by PCR using 5' sequence primers will contain *Ac* sequences in mutants, and a deletion or insertion of the DNA at the site of *Ac* insertion in *Ac*-deficient mutants when compared to the amplified DNA of this region in wild type. The presence of a mutant footprint in *Ac*-deficient mutants will be conclusive proof that the *arc*11 mutant was induced by and tagged with an *Ac*.

The isolation of somatic revertant sectors in *arc*11 individuals illustrates one of the benefits of a sub-cellular mutant phenotype in this work, since the *arc* mutant phenotype allows defined areas of revertant tissue to be observed. Somatic reversion has been assumed in non cell-autonomous mutant phenotypes by the evidence of the partial restoration of wild type plant phenotype (Bancroft, Jones and Dean, 1993), however the precision of this phenomenon has not yet been noted in *Arabidopsis* as it has in maize or *Antirrhinum* (Langdale, 1995).

The presence of somatic revertant sectors in an *arc* mutant also illustrates the cellular autonomy of the *arc* mutant effect. This autonomy has been noted between leaf cell types, such as the lack of a mutant chloroplast phenotype in *arc*1 parenchyma sheath cells

(Pyke and Leech, 1994), but the revertant sectors in *arc*11 are the first result showing both mutant and wild type phenotypes between neighbouring *mesophyll* cells of a single leaf.

The low incidence of reversion of the *arc*11 phenotype due to excision of the Δ*Nae*I *Ac* element allows for the effective use of *arc*11 individuals in genetic and phenotypic studies of the *arc*11 gene. However the potential for reversion of the mutant phenotype must be considered, requiring sufficient complementary duplicate experiments to be performed in genetic or biochemical analyses to reduce this potential inaccuracy.

The preliminary investigation of the putative sequence of the *ARC*11 gene has been undertaken by the analysis of small fragments of plant DNA flanking the *Ac* insertion. 350bp of the 1.1kp 3' fragment and the 150bp 5' fragment have been sequenced and screened for homology to catalogued nucleotide and amino acid sequences in other species. Neither of the fragments analysed demonstrates homology of the six polypeptide sequences predicted from the nucleotide sequence (three frames, each with forward and reverse orientation) to documented sequences. The small size of the fragments isolated may reduce the potential for reliably identifying homology in the protein sequence due to the small size of the predicted polypeptide sequence from a 349bp or 150bp fragment. Alternatively there may be no homologous polypeptide sequences to the *ARC*11 gene identified at present.

The extremely high degree of homology of the nucleotide sequence of the 1.1kb 3' fragment to that of an *E. coli* gene may justly prompt concern as to the origin of the cloned and sequenced 1.1kb 3' IPCR fragment. The lack of homology of the 1.1kb fragment to plant DNA in figure 6.8*(a)* also suggests that it may not be reliable as an *ARC*11 sequence. Since two fragments were amplified by the 3' IPCR it is likely that the other, 0.8kb, fragment is representative of the *ARC*11 gene, whist the 1.1kb fragment was amplified by the *Ac* from an origin other than the plant DNA. How this may have occurred is unknown at present. The analysis of the nucleotide sequence of the 0.8kb fragments and the verification of its hybridisation to the Southern blots of figure 6.8 will verify whether the 0.8 3' fragment contains *ARC*11 gene sequences or whether it, too, is more likely to be another type of DNA, as is probably the case with the 1.1kb 3' fragment.

The use of transposon mutagenesis for the isolation of *arc* mutants by gene tagging has been successful in the isolation of at least one fragment of the *ARC*11 gene. This *ARC*11 nucleotide sequence may now be used either to synthesis PCR primers to amplify other regions of the *ARC*11 gene or as probes for clones of *ARC*11 in cDNA or genomic libraries. The *arc*11 mutant was the only *arc* mutant identified from the seeds of the transposon mutagenised screened population and is tagged with a functional DNA insertion. In contrast, the screen of the T-DNA mutagenised population yielded six mutant individuals, none of which was tagged with a functional T-DNA insert. This result may suggest that the mutagenesis of plant genes by the use of a transposon is more reliable than the use of a T-DNA, however this evidence is only circumstantial; few mutants have yet been isolated from the transposon mutagenised lines of Dean and co-workers and, therefore, the frequency of tagged mutants may not be compared to that of the Feldmann T-DNA lines at this time.

Transposon mutagenesis has several advantages over the T-DNA mutagenised populations. The isolation of tagged mutants by IPCR is more reliable than by plasmid rescue, since the IPCR reaction only requires the uncorrupted presence of two short primer sequences, of which several exist for each border. By comparison, plasmid rescue requires more substantial pBR322 and border sequences to remain intact for the self replication of tagged DNA fragments. The transposon populations have been pre-screened for somaclonal variation before the transposition event to remove obvious background mutations. Therefore, whilst some subtle mutations may remain, the majority of mutations observed in screens of generations after transposition will be due to insertional mutagenesis of the transposon rather than background mutagenesis. The T-DNA populations have been observed to carry a high incidence of background mutation (Castle, Errampalli, Atherton *et al*, 1993).

In other aspects the transposon mutagenised lines are less efficient for the isolation of tagged mutants than the T-DNA populations. For example, the T-DNA mutagenised population bears a selectable marker for the T-DNA (kanamycin resistance) and verification of the tagging of a T-DNA mutant by a kanR phenotype is considerably easier than the use of Southern blotting to determine the presence of an *Ac* element.

Furthermore, the T-DNA insertion is stable and not prone to unpredictable excision observed in the transposon mutagenised individuals. The tendency of transposons to reinsert into local regions close to their original locus also additionally reduces the efficiency of the spread of mutagenised loci across the genome in these populations compared to the T-DNA populations. However the production of an increasing number of lines which contain mapped *Ds* elements which can be used for site- directed localised mutagenesis of mapped loci is of value to the construction of tagged mutant individuals.

The characterisation of the *ARC*11 gene will greatly facilitate further study of the gene's effect in *Arabidopsis*, and may also indicate several potential parallels between the function of *ARC*11 and other *ARC* genes. The isolation of *ARC*11 gene sequences by the amplification of an *Ac*-tagged locus has therefore provided a rapid advancement towards the eventual understanding of the function of *ARC*11 and clearly demonstrates the potential for the use of gene tagging by transposon mutagenesis in *Arabidopsis*.

Future work

The primary objective for future work with *arc*11 is the verification of the tagged nature of *arc*11. The use of probes constructed from the sequence data of the IPCR fragments will facilitate the amplification of the region of *ARC*11 which spans the locus of *Ac* insertion. This region in the mutant individuals not bearing an *Ac* is anticipated to demonstrate evidence of an excision footprint when compared to the sequence of the region in wild type. This ambiguity must be resolved before further analysis of *ARC*11.

Provided the tagged nature of the *arc*11 mutant is verified, the characterisation of the complete *ARC*11 gene may proceed by the isolation of a clone of the *ARC*11 gene either from a genomic or cDNA library or by PCR using probes from the *ARC*11 gene sequences isolated by IPCR of mutant DNA. The comparison of genomic and cDNA sequences will provide evidence of the open reading frames of the *ARC*11 gene and is anticipated to facilitate the prediction of the ARC11 protein sequence. The full *ARC*11 gene sequence may also be used in a complementation test to restore *ARC*11 gene activity in *arc*11 mutants by transformation of the mutant material with *ARC*11 gene sequence under a suitable promoter.

An additional approach to the characterisation of the function of the *ARC*11 gene is the analysis of the temporal and spatial co-ordination of the transcription and translation of the *ARC*11 gene sequence. The techniques adopted by Marrison and Leech (1994) for labelling of sectioned leaf tissue and M. Bennett (personal communication) for the labelling of whole tissue with DIG-labelled riboprobes or DNA probes will provide further understanding of the developmental co-ordination of chloroplast replication. The ontogenetical gradient evident in the first leaf of wheat *(Triticum aestivum)* cv. Maris dove (Marrison and Leech, 1992) or cv. Hereward (Robertson and Leech, 1995) will provide an ideal system for this study, provided that the *Arabidopsis ARC*11 gene is sufficiently homologous to that of wheat.

6.4 SUMMARY

The *Ac* transposon-mutagenised *arc*11 mutant displays a chloroplast mutant phenotype of c.25% of the number of chloroplasts in wild type and a chloroplast size of between half and ten fold larger than wild type. The chloroplast complement of *arc*11 mesophyll cells is highly variable in chloroplast size and number, both within and between individual mesophyll cells.

Analysis of the cosegregation of the *arc*11 mutation with the *Ac* element indicated that only 59 out of 66 F2 mutant individuals of a backcross to wild type contained an *Ac*. The analysis of 1200 mutant seedlings for revertant sectors, however, revealed 18 seedlings which showed evidence of somatic reversion; one of which showed evidence of germinal reversion in its progeny. The somatic and germinal revertants are taken as proof that the *arc*11 mutant is tagged with a functional *Ac* element. *ARC*11 gene sequences flanking the *Ac* were isolated by IPCR; cloned and sequenced. The DNA sequence which flanks the 3' end of the Ac demonstrates 99.8% homology to the *E. coli* rcsB and rcsC genes for capsule synthesis, but is unlikely to be a fragment of the *ARC*11 gene. The 5' flanking DNA demonstrates no nucleotide sequence homology to presently characterised genes.

CHAPTER 7

General Discussion

7.1 *arc* mutants of *Arabidopsis*

The analysis of *arc* mutants of chloroplast number and size in *Arabidopsis* has revealed several independent nuclear loci which affect the accumulation of the chloroplast complement in higher plants. The high number of *ARC* loci suggests that the control of chloroplast accumulation in *Arabidopsis* mesophyll cell is a very complex process involving the co-ordination of several independent cellular factors.

The isolation of *arc* mutants in *Arabidopsis* has altered the perspective of the analysis of the control of chloroplast division in higher plants. The major limitation to the study of chloroplast division has previously been the necessary reliance on circumstantial observations of the dividing chloroplast. Observations of physical events in chloroplast division have advanced the understanding of chloroplast division to a considerable extent, but do not provide precise indications as to the biochemical nature of the processes involved. The isolation and characterisation of genes which, directly or indirectly, affect chloroplast division and expansion is anticipated to lead to a more definite understanding of the precise processes underlying the microscopical observations of chloroplast division in higher plants.

The use of mutagenesis in the study of the role and function of *ARC* genes has provided a null baseline of individuals which do not undergo chloroplast division, i.e. *arc*3, *arc*5 and *arc*6. This null effect of a complete inhibition of chloroplast division may be compared to less extreme reductions in chloroplast number such as the phenotype of *arc*11 or *arc*2, or to different phenotypes which enhance division, such as the *arc*1 and *arc*7 effects. The theories which arise from the study of mutant phenotypic traits are, however, mostly speculation. *ARC* gene isolation was therefore employed to provide the analysis of chloroplast division with defined gene products and gene effects.

The isolation of *ARC* genes has been central to the work of this thesis, and the value of mutagenesis is of clear benefit here. The gene isolation process of 'forward genetics' in which the gene product is isolated, sequenced and the gene sequence deduced is clearly of little use to the analysis of *ARC* genes. The complexity of the control mechanisms combined with the total lack of understanding of the products involved in the chloroplast division process in mesophyll cells would make the isolation of ARC proteins

extremely difficult and unreliable. The approach of 'reverse genetics' involving the isolation of a mutant gene from which a polypeptide sequence may be deduced is a more suitable approach for the study of chloroplast division. The use of insertional mutagenesis is also of considerable benefit since the isolation of a tagged locus is likely to shorten the process of gene isolation by several years compared to a map-based cloning strategy. The isolation of a tagged mutant, *arc*11, is potentially very significant; the *arc*11 mutant is not the most extreme mutant phenotype isolated, but is a phenotype which displays a significant perturbation to the chloroplast division process. Knowledge of the exact role of the *ARC*11 gene will aid further understanding of the action of other *ARC* gene effects.

The co-ordination of the accumulation and replication of chloroplasts in the mesophyll cells of *arc* mutants shares several remarkably consistent traits between diverse mutant phenotypes. A constant relationship between the total chloroplast area and increasing mesophyll cell size is observed in every mutant from the single large chloroplast phenotype of *arc*6 to a mutant with numerous small chloroplasts, such as *arc*1. This effect, due to a compensation of chloroplast size for deviations in chloroplast number illustrates the extreme plasticity of the process of chloroplast division and expansion in *Arabidopsis*.

Surprisingly, the majority of the *arc* mutants do not display a significant whole plant mutant phenotype, suggesting that an alteration of the development of the chloroplast complement is not significantly deleterious to the function of the *Arabidopsis* mesophyll cell. The high degree of variation which may occur in the development of the chloroplast population of *Arabidopsis* questions the need for chloroplast division in *Arabidopsis* mesophyll cells. Whether this variation in the composition of the chloroplast population is tolerated by the mesophyll cells of other species than *Arabidopsis* is not known. It is possible that in species such as wheat or maize such variations in the chloroplast population as are seen in the *Arabidopsis arc* mutants would result in more deleterious effects than have been observed.

The high (but not absolute) degree of uniformity of the chloroplast population *between* cells in both wild type and *arc* mutants of *Arabidopsis* (shown in 3.2.2) and the extreme phenotype of many *arc* mutants illustrates the high degree of refinement which

affects the process of chloroplast accumulation in *Arabidopsis*. The development of the chloroplast population is likely to be a product of the co-ordinated effect of several independent cellular processes - some of which affect the plastid directly, some of which are probably incidental to plastid division. The apparent complexity of chloroplast division illustrates how highly integrated the process is into the development of the mesophyll cell. The value of further study of the accumulation of the chloroplast population to the understanding of higher plant cellular development is considerable, especially in the consideration of the genetic influences which control mesophyll cell development

7.2 The Physical Controls to Chloroplast Division in Higher Plants

The effect of physical factors in the cell on the accumulation of the chloroplast population in *Arabidopsis* is likely to be one of the principal mechanisms governing the phenotype of the chloroplasts. The effects of plastid size and cell size (section 4.2.4 and 4.2.5 respectively) are the most significant of these controlling factors, both as positive and negative controls of the accumulation of plastids in the cell. There is also likely to be a dependence on defined criteria for the replication of *Arabidopsis* chloroplasts, such as the optimum chloroplast size for division (4.2.4) and the limitation induced by cell size on the replication and expansion of the chloroplasts (4.2.5). Other criteria have been cited as the primary factors in the control of the accumulation of the chloroplast complement in higher plants, primarily nuclear and chloroplast DNA amount and light intensity and quality. These factors must not be discounted, although they are not likely to be principal factors in the cellular definition of the chloroplast complement (discussed in 1.4). They are, however, important adaptive effects which may alter the balance of the more direct physical constraints so that stress or changes in growth conditions are countered by altered chloroplast development as the cell expands.

The initiation of chloroplast division in *Arabidopsis* may follow a similar process to that of wheat (Boffey, Ellis, Sellden and Leech, 1979). The expansion of post-meristematic mesophyll cells at the base of the wheat leaf stimulates the division of the young green chloroplasts which proceeds over a short period of subsequent cell

expansion. Boffey, Ellis, Sellden and Leech (1979) note that the first cycle of chloroplast division in wheat is synchronised, which suggests a cellular control process. The initiation of division in an increased proportion of chloroplasts is observed in the youngest mesophyll cells of wild type *Arabidopsis*, *arc*5 and *arc*1/*arc*5 double mutant suggesting a moderately similar mechanism is in operation. The increase in chloroplast number in both wheat and *Arabidopsis* is co-ordinated with the increase in cell size of the young mesophyll cells (Pyke and Leech, 1987 and 1991; figure 4.7). The initiation of chloroplast replication may therefore be a spontaneous process which reacts to the sudden availability of cellular space into which the chloroplasts may accumulate.

The analysis of division profiles in wild type *Arabidopsis*, *arc*5, the *arc*1/*arc*5 double mutant and also in wheat (Ellis and Leech, 1985) have suggested that there is an optimum chloroplast size at which division occurs (4.3). In *Arabidopsis* ecotype Landsberg *erecta* this optimum is approximately 50μm^2, in wheat the optimum size is 6-10μm^2. The division of the chloroplast reduces its plan area by approximately half, requiring the subsequent further expansion of the chloroplast before division occurs again. The analysis of the proportions of wild type *Arabidopsis* chloroplasts to undergo division (figure 4.7) suggests that only a maximum of 26% of chloroplasts are in division at once, even during the initial rounds of plastid replication. The lack of synchrony suggests that not all chloroplasts in Landsberg *erecta* wild type attain the optimum size together. The analysis of the plan areas of dividing chloroplasts also indicates that not all chloroplasts divide at the optimum size and that, within limits, chloroplast division may also occur, to a lesser extent, in smaller or larger plastids. This deviation from the mean would explain the lack of completely synchronous division in the chloroplast population. The proportion of plastids in division in wheat is greater than is observed in *Arabidopsis*, however chloroplasts of wheat are considerably smaller than those of *Arabidopsis*; the variation in the size of dividing chloroplasts would therefore be reduced, resulting in more uniform division.

The lack of co-ordination between individual chloroplast divisions due to the individuality of the division process in each chloroplast is further supported by the variable chloroplast phenotypes of the mutants *arc*2 and *arc*11. The most probable

explanation for these phenotypes is that division has occurred in different chloroplasts at different stages of their expansion. The timing of each chloroplast division within a lineage of these mutant chloroplasts will indicate the final size of each plastid when cell expansion has stopped. Those chloroplasts which were unable to divide in earlier cell development would become the larger plastids of the population, whereas those which continued to divide throughout cell expansion would be smaller.

The continual cycle of division in chloroplasts which are of a similar size to each other in the expanding cell indicates that chloroplast expansion is a continuous process, increasing the size of daughter plastids until they may undergo a subsequent division. This observation is supported by the mutants *arc*3, *arc*5 and *arc*6 in which chloroplast number does not increase, since the chloroplasts of these mutants expand to counteract the deficiency in plastid division. The expansion of the chloroplast is an extremely plastic process, demonstrated elegantly by the chloroplast number and size of the *arc*6 mutant, and is vital to the accumulation of the chloroplast population as the mesophyll cell expands. The accumulation of the chloroplast population may therefore be a continual competition between chloroplast expansion and chloroplast division. As the chloroplast expands, it reaches the optimum size for division to occur, whereupon it is split into two daughter plastids of a reduced size, requiring expansion again before subsequent division. If the chloroplast divides early, as may happen in *arc*1 or *arc*7, the plastid does not accumulate the wild type size, resulting in a population of smaller chloroplasts than wild type. Should division not proceed or is not efficient enough to divide the plastid before the maximum size for division is passed, the chloroplast will continue to expand until restricted by another factor, such as cell size.

The mechanisms controlling the initiation of the various stages in plastid division proposed above strongly imply that the plastid division process is a spontaneous event initiated by the co-ordination of several independent physical phenomena. The initiation of plastid division may therefore not be a distinct process initiated by the cell during development, but rather an opportunistic process which will proceed spontaneously if the conditions are correct unless restrained by the cell. Such a spontaneous process would be supported by the observations of researchers such as Ridley and Leech (1970) that the

division of isolated plastids may occur in an artificial environment, removed from the control of the nucleus. A spontaneous initiation of the constriction of chloroplasts would require a process which was independent of cell metabolism, such as the theory based on the physical observations of Greenspan (discussed in 1.3.2). The initiation of constriction by physical properties of the chloroplast, such as size and tension of the envelope membrane, would provide a mechanism to explain the independent rate of individual chloroplast divisions observed in both wheat, spinach and *Arabidopsis*. The reliance on size constraints for the initiation of chloroplast division in *Arabidopsis* suggests that there is a competition between chloroplast expansion and chloroplast division during the accumulation of the chloroplast complement in mesophyll cells.

The association between chloroplast number and mesophyll cell size has been observed in many species (reviewed in 1.4.1). Such an association, across a broad range of cell sizes, would suggest that the mesophyll cell restricts the chloroplast population to a defined proportion of its volume. The chloroplast complement, once it had filled this proportion of the cell, would be inhibited from increasing in volume until cell expansion proceeded further. Chloroplast number per cell is therefore not a pre-determined quantity, but rather a process which is regulated indirectly by the steady expansion of the cell in tandem with leaf expansion. This mechanism is possibly a parallel of the host/endosymbiont relationship between the evolutionary precursor of the chloroplast and the early plant cell. The cell would provide an environment for the growth and reproduction of the endosymbiont, in return for photosynthetic products. The over-accumulation of the endosymbiont would endanger the viability of the host cell despite the potential for increased photosynthetic yield and therefore would be restricted by the cell. This requirement for a control of an endosymbiont is observed in the cells of the green *Hydra* which contain a population of symbiotic *Chlorella* algae. Investigations of the accumulation of the numbers of *Chlorella* in *Hydra* cells by McAuly (1981) demonstrates a rigid control by the host cell to the division of *Chlorella* cells, restricting their replication to mitotic *Hydra* cells. Although the precise nature of this restriction in endosymbiont replication is unclear, the rigid control of symbiont replication by the host implies that abnormally increased numbers of the symbiont are undesirable to the host cell.

Chloroplast expansion may therefore continue for as long as it is able, being regressed occasionally by chloroplast division, but once the cellular maximum is reached, both division and expansion of the chloroplasts is forced to cease. The rate of cell expansion is therefore an important control to the accumulation of the chloroplast complement for each individual cell. Cell expansion itself may be altered, either by changes in cell ploidy (Pyke, Jellings and Leech, 1990) or by chemical treatment (Leech and Pyke, 1988), but the chloroplast population still continues to be restricted by the size of the cell.

7.3 The Genetic Control of Chloroplast Division in *Arabidopsis*

The control of chloroplast division in *Arabidopsis* also involves the effects of genetic mechanisms as well as physical criteria. The primary consideration in the analysis of the genetic control of chloroplast division is how the *ARC* genes may affect the division process. The most likely explanation would be the synthesis of products which are directly involved in the division process, for example the components of the plastid dividing ring. However, the high number of *ARC* loci and the variety of *arc* mutant phenotypes suggests that not all the *ARC* genes identified by mutation directly affect the process of chloroplast division. The most probable effect of many *ARC* genes on the accumulation of the chloroplast complement is an indirect effect. The only *arc* mutation which would appear to affect directly chloroplast replication is the *arc*5 mutation which appears to arrest chloroplast division before it is completed (discussed in 4.3). It may be envisioned that the combined effect of several *ARC* genes results in the phenotype of the overall chloroplast complement. The refinement of the co-ordination of these several incidental processes over evolutionary time would result in a relatively consistent chloroplast population for each individual species or ecotype.

The *ARC* genes appear to affect the plastid population at several different levels. Firstly the control of the completion of the division process would appear to be affected by the *ARC*5 gene. The efficient completion of the process of division is of considerable significance when set against a background of continuous chloroplast expansion. The expansion of the chloroplast beyond the maximum limit of size for division to proceed

results in a low number of large chloroplasts as a cellular complement. The initiation of chloroplast division would appear to be directly affected by the *ARC*3 and *ARC*6 genes (and possibly the *ARC*2 or *ARC*11 genes), the absence of which retard the initiation of division in either proplastids, chloroplasts or both sufficiently for the plastid to expand beyond the limits of successful division. The initiation of division is also indirectly affected by the *ARC*1 and *ARC*7 gene effects, a lesion which through some means stimulates division at an earlier stage than in wild type. The precise role of the *ARC*1 and *ARC*7 genes to mesophyll cell function is unclear, but may possibly be an effect on the synthesis of lipid constituents in the cell. The moderate phenotype of the *arc*4 and *arc*8 mutants suggest the likelihood that the respective *ARC* genes affect less specific functions to chloroplast division.

The complexity of the co-ordination of the process of chloroplast division suggests that few of the *ARC* genes may be accurately described as genes for chloroplast division, but rather as genes which *affect* chloroplast division. The frequency of *arc* mutations in the screens of the mutagenised populations suggests that numerous *arc* mutant phenotypes have yet to be isolated. The several *ARC* loci, which are likely to affect a number of different cellular processes demonstrate the value of the understanding of chloroplast accumulation and replication to the study of cellular development in higher plants.

7.4 A Model for Chloroplast Division in *Arabidopsis*

The analysis of the action of *arc* mutations on the process of chloroplast division allows a tentative model for the control of chloroplast division in *Arabidopsis* to be proposed, illustrated in figure 7.1. This model is largely reliant on speculation, based on observations of mutant phenotypes, and is intended more for discussion than as a working theory.

(i) The division of proplastids.

The proplastids of the meristematic cells in *Arabidopsis* appear to divide in association with the nucleus. The proplastids of the meristematic cell probably divide during mitosis. The ***ARC6*** gene affects the division process in the proplastid, most

FIGURE 7.1 A Model for Chloroplast Division in *Arabidopsis*

(a) The small, undifferentiated proplastid undergoes division in the meristematic cells.

(b) Initiated by the *ARC*6 gene, the proplastid is constricted about its equator. Proplastid expansion continues throughout the division process.

(c) The constricted proplastid divides into two daughter plastids. The daughter proplastids are segregated into daughter cells at cytokinesis.

(d) Proplastid division continues in association with cell division until meristematic activity stops. *c.*14 proplastids are allocated to each post-meristematic cell.

(e) The young chloroplast develops thylakoid membranes; *ARC*1 and *ARC*7 are probably involved in the association of the protein complexes into the thylakoids.

(f) The developing chloroplast expands in tandem with mesophyll cell expansion.

(g) The chloroplast reaches 25μm^2 in plan area; chloroplast division may occur in the plastid at this size but is unlikely to do so until the chloroplast is larger. The *arc*1 and *arc*7 mutant chloroplasts divide optimally at this chloroplast size.

(h) The chloroplast reaches 50μm^2. This is the optimum size for division to be initiated. If the chloroplast fulfils the requirements of *(i)* and *(j)* below, division proceeds.

(i) If there is sufficient space available in the proportion of the cell which has been allocated to the chloroplast complement, division will occur.

(j) Chloroplast expansion is continuous throughout the cycles of chloroplast division. If the chloroplast is larger than *c.* 65μm^2 division is unlikely to be initiated and the chloroplast will expand instead to fill the requisite chloroplast area per cell.

(k) The *ARC*3 gene (and possibly other *ARC* genes) facilitate the initiation of a chloroplast division. Invagination of the chloroplast envelope is due to differential tension forces over the envelope surface causing constriction at the equator.

(l) The invagination of the chloroplast equator facilitates the association of the plastid dividing ring. The plastid dividing ring, probably an actin/myosin structure, causes further constriction when the isthmus has narrowed to c.30% of the plastid diameter.

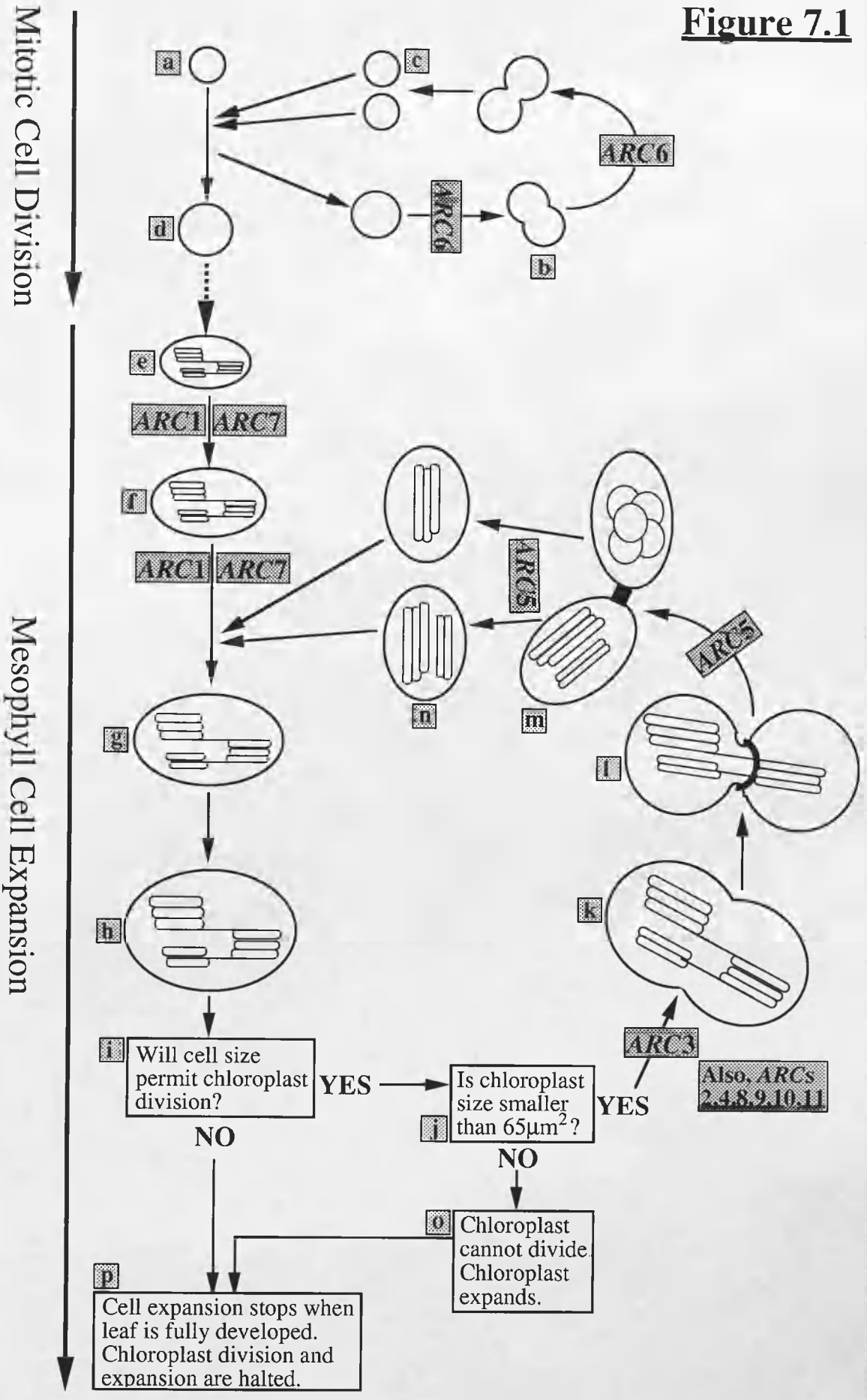
(m) The plastid dividing ring wraps around itself constricting the isthmus diameter. The *ARC*5 gene facilitates this function, possibly coding for a constituent of the ring. The two halves of the constricted plastid twist and bend at the isthmus, often by more than 90°. The plane of section of the flat thylakoids is shown in the constricted plastid.

(n) When the plastid dividing ring has constricted the isthmus to the maximum extent, the envelope membranes at the tightly constricted isthmus fuse and plastokinesis occurs.

(o) Chloroplasts which do not initiate or complete a division before the maximum size for division is reached, expand instead of dividing to fill the required chloroplast area.

(p) The chloroplast population is restricted to a defined proportion of the cell volume during mesophyll cell expansion. When cell expansion stops both chloroplast division and expansion are halted.

Figure 7.1


Mitotic Cell Division
Mesophyll Cell Expansion
a
c
ARC6
d
ARC2
b
e
ARC1
ARC7
f
ARC1
ARC7
ARC6
ARC5
n
m
g
l
h
k
i
Will cell size permit chloroplast division?
YES
Is chloroplast size smaller than 65µm²?
j
YES
ARC3
Also, ARCs 2,4,8,9,10,11
NO
NO
o
Chloroplast cannot divide. Chloroplast expands.
p
Cell expansion stops when leaf is fully developed. Chloroplast division and expansion are halted.

probably affecting the initiation of proplastid division. The proplastids, due to their association with the nucleus, are apportioned equally during cytokinesis to the poles of the dividing cell. This compartmentalisation may rely on the even distribution of the proplastids around the nucleus for the equal segregation of proplastids into daughter cells. The process of segregation of proplastids is not, however, exact since although a common number of 14 proplastids per cell is normal in *Arabidopsis*, this number may vary slightly due to the random distribution of the proplastids about the nuclear surface.

(ii) The division of chloroplasts

The un-vacuolated, post-meristematic cell begins to expand as the leaf primordia develops into the lamina. The cytoplasmic compartment of the cell begins to increase in volume. Prior to this increase the division of the young green chloroplasts is inhibited due to their small size and to the lack of space in the cytoplasm not allowing for further expansion of the chloroplast complement. The expansion and vacuolation of the young mesophyll cell, and the provision of resources for chloroplast division and expansion enables the growth and development of the young chloroplasts as well as the other cytoplasmic components of the cell. The ***ARC1*** and ***ARC7*** genes may have an indirect effect on this process, allowing for the efficient production of the chloroplast membranes and the correct constitution of these membranes. The accumulation of the thylakoid system within the chloroplast may be affected by these genes as may be the stability of the envelope membranes. A lesion in either of these genes is likely to lead to the retarded expansion of the plastid and the retarded integration of the photosynthetic apparatus into the thylakoid membranes. A lesion in the *ARC*1 and *ARC*7 genes may possibly induce premature initiation of the division of the chloroplast during chloroplast expansion. The expansion of the chloroplasts will proceed more or less indefinitely until it is restricted by another factor such as chloroplast division, cell size or a restriction in resources.

Once an optimum chloroplast size of approximately 50μm^2 is reached the chloroplast division process is spontaneously initiated by the interaction of surface tensions on the chloroplast envelope. The reduction of tension at the poles of the chloroplasts initiates the formation of a furrow at the equator of the plastid, allowing

constriction to proceed. The ***ARC3*** (and possibly also ***ARC6***) genes may affect this process, aiding the initial disturbance in tension at the chloroplast poles. A lesion in these genes would cause a severe retardation of the initiation of division such that, all other factors being equal, the chloroplast will not divide before plastid expansion has removed the potential for division.

The initiation of chloroplast constriction or separation is also likely to be affected in a more moderate manner, possibly due to an indirect effect, by the ***ARC2***, ***ARC8***, ***ARC9***, ***ARC10*** and ***ARC11*** genes. The action of these genes may stimulate division in a variety of circumstances, so that a lesion in their action would reduce the efficiency of constriction in some plastids, leading to a variable population of plastid sizes and shapes. It is possible that the extremity of the *arc* phenotype in mutants of these genes represents the timing of the gene effect in cellular development. The ***ARC9*** and ***ARC10*** genes, for instance, may only affect the initial cycles of chloroplast division, so that two distinct populations of chloroplasts are formed in the mutants of these genes, those which missed the initial divisions and expanded subsequently, and those which have completed division throughout cellular development. The ***arc2*** and ***arc11*** mutants may represent lesions of similar effects, but with a more prolonged duration, resulting in a more varied phenotype. The ***ARC8*** gene is likely to have a minor effect on the process throughout development, hence the more uniform chloroplast phenotype of the *arc*8 mutant.

Once initiated, the process of constriction proceeds rapidly and the isthmus progressively reduces in diameter. The spontaneous constriction of the plastid at the equator is aided and completed by the association of a plastid dividing ring with the outer and inner envelope membranes. The nature of this association is unclear, as is its timing, although the ring is likely to be an actin based structure, possibly part of the cytoskeleton or plastid skeleton. The plastid dividing ring is first resolvable when the isthmus has constricted to approximately a third of the plastid diameter; however it is likely that the ring is functional before it is resolved by the microscope. The spontaneous constriction of the plastid is predicted to cause a streaming of the stromal constituents leading to a concentration of material in the region of the isthmus. This concentration may accumulate sufficient quantities of filamentous material on the cytoplasmic and stromal surfaces of the

envelope for an association to be formed at the axis of the constriction, leading to the functional plastid dividing ring. The plastid dividing ring is a structure of defined length so that the means of constriction of the isthmus is by the plastid dividing ring wrapping around itself. This effect may be analogous in nature to the action of the actin/myosin association in contracting mammalian muscle fibres. The function of the plastid dividing ring is affected by the ***ARC5*** gene, so that a lesion in *ARC5* reduces the efficiency of the plastid dividing ring's function - possibly its ability to associate with and bind to itself. The plastid dividing ring may constrict to a significant degree in *arc5* mutants, but does not constrict sufficiently for the separation of the daughter plastids to proceed. Furthermore, when subjected to subsequent chloroplast expansion, the plastid dividing ring of an undivided *arc5* mutant plastid may unravel, allowing the isthmus to expand once more with the inflating chloroplast. Once the isthmus is fully constricted the envelope membranes at the constricted region fuse and plastokinesis is completed.

(iii) The limitation of chloroplast division and accumulation

The chloroplast division process is set against a background of continual chloroplast expansion. As the chloroplast divides it is separated into two daughter plastids of more or less equal size, approximately half the size of the parent. The daughter plastids then expand until the optimum size for division is reached a second time before dividing once more. The combined effect of small differences between the rate of expansion, the size at which division is initiated and the speed of the division process causes each individual chloroplast to proceed with the division cycle at an asynchronous rate to its neighbouring plastids. This individuality demonstrates the degree to which the division process is reliant on the physical phenomena surrounding the individual plastid.

Plastid division and expansion proceed until the proportion of the cell allocated to the chloroplast population is filled. As the cell expands during leaf development, the volume of cell space allocated to the chloroplasts also increases in proportion and division occurs in those plastids for which the conditions are correct. In this manner a steady number of chloroplast divisions are maintained throughout mesophyll cell development, until cell expansion is complete, whereupon chloroplast division ceases.

7.5 Summary

The isolation of *arc* mutants of chloroplast number and size in *Arabidopsis* has provided a significant advancement to the study of chloroplast division in higher plants. Prior to the characterisation of *arc* mutants, investigations of chloroplast replication based on microscopical observations were limited largely to speculation as to the nature of the events involved in the division process. Whilst the use of the *arc* mutant phenotypes themselves is subject to similar limitations at present, the potential benefit provided by *arc* mutants is considerable. The phenotype of those *arc* mutants in which division is completely suppressed provides a null baseline of mesophyll cells in which the chloroplasts do not replicate. Plastid morphogenesis in such mutant cells may therefore be related to that of normal tissue and the progression of events involved in plastid division in the two systems may be compared. The observation of the physical events in chloroplast division may therefore be focussed and defined more clearly.

arc mutants demonstrate clearly the broad extent to which the cell can tolerate extreme lesions in the accumulation of its chloroplast population. The *arc* mutants display a very wide range of plastid number, size and shape, yet very few of the mutants shows any significanty deleterious effect on the development of the whole plant. The existance of normal, healthy, fertile plants which may have, as is the case in *arc*6, as few as a single chloroplast per mesophyll cell has considerable repercussions to the perception of the development of the plant cell and its components.

The most valuable asset of the *arc* mutants is in the isolation of the *ARC* genes which control or affect chloroplast replication. The use of mutagenesis to isolate *ARC* genes by reverse genetics will enable each aspect of chloroplast division to be studied in precise detail. In the short term the characterisation of *ARC* genes is anticipated to provide information concerning several independent processes, each of which is concerned with chloroplast division. In the longer term, however, the understanding of the interaction of numerous *ARC* gene effects both with each other and with the metabolism of the cell, will provide considerable insight into the process of higher plant cell development and the co-ordination of the many and complex sub-cellular systems in higher plant cells.

CHAPTER 8

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APPENDIX A
Origin of Chemicals

Chemical	Origin	Stock Number
α^{32}P dCTP radioisotope label	NEN Dupont	NEG013-H
Acetic acid CH_3COOH	BDH	10001
Agarose	Sigma	A-9539
Ammonium acetate (**NH_4Ac**) $NH_4C_2H_3O_2$	Sigma	A-1542
Bacto Agar	Difco Labs	0140-01
Boric acid H_3BO_3	BDH	10058
Bovine Serum Albumen (**BSA**)	Sigma	B-4287
Bromophenol Blue	Sigma	B-5525
Calcium chloride $CaCl_2$	Fisons	C/1500
Chloroform $CHCl_3$	BDH	10077
4,6-Diamidino-2-phenylindole (**DAPI**) $C_{16}H_{15}N_5.2HCl$	Sigma	D-9542
Diaminoethanetetra acetic acid (disodium salt) (**Na_2EDTA**) $C_{10}H_{14}N_2O_8Na_2.2H_20$	Fisons	D/0650
Diglycidyl ether of polypropylene glycol (**DER 736**)	Sigma	D-8165
Ethanol C_2H_5OH	BDH	10107
Ethidium bromide $C_{21}H_{20}N_3Br$	Sigma	E-8751
Ficoll	Sigma	F-2637
Formvar	Sigma	F-6146
Glutaraldehyde (AR grade) $C_5H_8O_2$	Sigma	G-6257
Glutaraldehyde (E.M. grade) $C_5H_8O_2$	Sigma	G-7526
Glycerol	Fisons	G/3107
Herring sperm DNA	Boehringer	22346
Iodine	Sigma	I-3380
Kanamycin $C_{18}H_{36}N_4O_{11}.H_2SO_4$	Sigma	K-4000
Lithium acetate (**LiAc**) $C_2H_3O_2Li.2H_2O$	Sigma	L-6883
Magnesium sulphate $MgSO_4$	Sigma	M-7506

Manganese chloride $MnCl_2.4H_20$	sigma	M-3634
Mixed Alkyltri-methylammonium bromide (**CTAB**) $C_{14}H_{29}N(CH_3)_3Br$	Sigma	M-7635
3-[N-morpholino] propanesulphonic acid (**MOPS**)	Sigma	M-1254
Nonenyl succininc anhydride (**NSA**)	Sigma	N-7644
Nutrient agar (Oxoid)	Unipath	CM3
Nutrient broth (Oxoid)	Unipath	CM67
Osmium tetroxide OsO_4	Sigma	O-5500
Paraformaldehyde (H.CHO)n	BDH	29447
Phenol C_6H_5OH	BDH	10188
Polyethylene glycol, 1500 grade (**PEG-1500**)	Fisons	P/13679
Polyvinylpyrolidone (**PVP**)	Sigma	PVP-10
Potassium dihydrogen orthophosphate KH_2PO_4	Fisons	P/4800
Potassium iodide KI	Sigma	P-8256
Propan-2-ol (**Isopropanol**) $(CH_3)_2CHOH$	BDH	29694
Rubidium chloride RbCl	Sigma	R-2252
Sodium acetate (**NaAc**) CH_3OONa	Fisons	S/2120
Sodium chloridc NaCl	BDH	10241
Sodium dodecyl sulfate (**SDS**)	Sigma	L-4509
Sodium hydroxide NaOH	BDH	10252
Sodium hypochlorite	BDH	
Spermidine	Sigma	S-2626
Sucrose $C_{12}H_{22}C_{11}$	Fisons	S/8600
Tri-sodium citrate $Na_3C_6H_5O_7.2H_2O$	BDH	
Triton X-100	Sigma	T-8787
Trizma base (**Tris**) $C_4H_{11}NO_3$	Sigma	T-1378
Trizma hydrochloride (**Tris HCl**) $C_4H_{11}NO_3.HCl$	Sigma	T-3253
Vinylcyclohexene dioxide (**VCD**)	Sigma	V-3630
Xylene Cyanol	Sigma	X-4126